MODULATION OF MITOGENIC SIGNALING AND GROWTH BY SYMPATHETIC ADRENERGIC REGULATION

BY

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DISSERTATION

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ABSTRACT

Heart disease and diabetes mellitus are growing epidemics, consistently ranking within the top ten causes of death in the United States. Both diseases are associated with cardiac remodeling, including cellular growth and fibrosis. Central to the progression of heart disease and diabetes are the adrenergic receptors (ARs) and insulin receptors (IR), respectively. Classically considered to elicit separate, and even opposing, cellular processes, increasing evidence suggests that ARs are capable of signaling cross-talk with other G-protein coupled receptors (GPCRs) as well as other receptor families, including receptor tyrosine kinases (RTKs). While the ARs have been implicated at numerous stages of heart failure, the adrenergic signaling mechanisms underlying this remodeling remain unknown. Further, studies investigating signaling cross-talk among different adrenergic receptors as well as between adrenergic receptors and other signaling pathways in myocardium remain in their infancy. In addition, few studies have implied functional interactions between IRs and βARs in cardiac tissues. Understanding how cells integrate information from a variety of chemically diverse signals into complex, orchestrated responses such as cell proliferation, differentiation and apoptosis is an overarching goal of cell biology. Thus, an understanding of the mechanisms and physiologic consequences of adrenergic receptor cross-talk within the heart is essential to develop novel treatments designed to prevent the cardiac remodeling observed in heart disease and diabetes. In concordance, the goals of this thesis research are two-fold. First, we aim to define mechanisms of AR cross-talk in cardiac cells. Secondly, we aim to determine the importance of these mechanisms in cardiac remodeling in response to pathological conditions.

To begin to address the goals presented we first examined cross-talk within GPCRs. We elucidated a general mechanism in which non-traditional GPCR signaling is capable of

regulating mitogen-activated protein kinase (MAPK) signaling originating from another GPCR. Interestingly, this cross-talk impaired GPCR-induced cellular proliferation. Next, we characterized a novel signaling mechanism in which type II RTK activation at high concentrations of mitogen can recruit non-traditional GPCR signaling components to fine-tune activation of MAPK signaling for cell proliferation. In sum, this work presents a significant expansion of our understanding of GPCR signaling cross-talk and its role in altering growth signaling originating from another receptor. Further, due to the complexities of hormonal signaling *in vivo*, this work highlights the need to further pursue a more in-depth understanding of how concomitant activation of one signaling pathway can alter the signaling and physiologic outcome of another receptor's signaling.

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Chapter 1:
Cardiac remodeling and the receptor signaling pathways implicated in its pathogenesis

Cardiovascular disease consistently remains the leading cause of morbidity and mortality in the United States, leading to nearly 30% of deaths in 2006. In addition to the human impact, the economic impact of cardiovascular disease is staggering with an estimated 503.2 billion dollars spent in 2010. Chronic diseases specifically affecting the heart, including heart failure, account for a significant portion of that impact (www.americanheart.org). Because heart failure increases the risk of sudden cardiac death six to nine times that of the general population, an understanding of the physiological and pathophysiological process of remodeling in the heart remains an important area of investigation. While the adrenergic receptors have been implicated at numerous stages of heart failure, the adrenergic signaling mechanisms underlying this remodeling remain unknown. Further, studies investigating signaling cross-talk among different adrenergic receptors as well as between adrenergic receptors and other signaling pathways in myocardium remain in their infancy. Importantly, no study has investigated the role of adrenergic receptor cross-talk in both physiological and pathological processes. Thus, the goals of this thesis research are two-fold. First, we aim to define mechanisms of receptor cross-talk in cardiac cells. Secondly, we aim to determine the importance of these mechanisms in cardiac remodeling in response to pathological conditions.

Cardiac remodeling and heart failure

Cardiac hypertrophy is an essential adaptive cardiac response initially serving as a compensatory response to increased work demand[1, 2]. This initial compensatory mechanism is the main indication for heart failure. Heart failure is the result of a well-characterized sequence of cardiac remodeling resulting from numerous environmental (hypertension, diabetes, ischemia) and genetic factors (congenital heart failure) (Fig 1.1). Hypertension, the leading risk factor for cardiovascular disease and heart failure, prevents efficient functioning of the heart and increases the preload of the left ventricle[3]. Increased preload in the left ventricle, along with elevated circulating catecholamines, leads to a compensatory increase in contraction strength via cardiac myocyte hypertrophy. This initial compensatory concentric cardiac hypertrophy is the leading indication for decompensated eccentric heart failure[4]. Increased thickness of the myocardium along with increased metabolic demand on the heart leads to endocardial ischemia and cardiac myocyte death. In an attempt to repair the damaged cardiac muscle, fibroblasts proliferate and secrete collagen, leading to cardiac fibrosis. Decreased myocardial mass, in addition to fibrosis, exaggerates the loss of cardiac contractile function leading to cardiac insufficiency; it also enhances cardiac arrhythmia and sudden cardiac death.

Although the mechanisms are unknown, cardiac remodeling and heart failure have been linked to chronic activation of the adrenergic receptors due to elevated circulating catecholamines[1]. While the functional integrity of adrenergic signaling is critical in maintaining cardiac output for physiological responses, decreased cardiac response to adrenergic stimulation is linked to the development of heart failure[5]. Further, adrenergic manipulation remains a leading treatment for hypertension and heart failure.

Diabetes and heart failure

Diabetes Mellitus is a growing epidemic, affecting nearly 24 million people, or nearly 8% of the population, in the United States. The human and economic cost of diabetes mellitus, the sixth leading killer in the United States, is enormous with direct diabetic care expenses totaling $116 billion dollars in 2007[6, 7]. Type 2 diabetes mellitus, accounting for 90-95% of diabetes cases, is a well known risk factor for cardiomyopathy and eventual heart failure (HF)[8, 9]. Diabetic cardiomyopathy (DCM) is characterized by both systolic and diastolic dysfunction resulting from aberrant contractility as well as cardiac hypertrophy, in the absence of coronary artery disease (CAD) and hypertension[10]. Interestingly, DCM is associated with hyperinsulinemia and depressed adrenergic signaling leading to decreased contractility despite elevated catecholamine levels, presumably due to either dysfunctional calcium handling within the cardiomyocytes or catecholamine-induced βAR down-regulation[11-14]. The lack of CAD and hypertension in DCM, even in the presence of elevated catecholamines, suggests that hyperinsulinemia elicits direct cardiomyocyte insult, possibly by modulating adrenergic function. Together, these data illustrate the potential importance of βAR/insulin receptor (IR) interactions in the hearts of diabetic patients. The mechanism and consequence of these interactions remains to be elucidated.

Adrenergic receptor structure and expression

The adrenergic receptors make up a class of heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs) that play a central role in mediating the effects of catecholamine hormones. GPCRs, the most ubiquitous receptors in the body, link ligand binding to G protein activation and signal transduction (Fig 1.2). The adrenergic receptors are

prototypical G protein-coupled receptors comprised of seven hydrophobic transmembrane helical domains with an extracellular amino terminus and an intracellular carboxyl terminus[15-18] (Fig 1.3). While the third, fifth and sixth transmembrane domains are essential for agonist binding, the third intracellular loop and C-terminus are important in G protein coupling[19-21]. Further, receptor desensitization and internalization is mediated by the intracellular carboxyl terminus[22, 23].

Nine subtypes of adrenergic receptors exist (β_1, β_2, β_3, α_1A, α_1B, α_1D, α_2A, α_2B, α_2C). These subtypes are widely expressed throughout the body. In humans the β_1AR is predominately expressed in cardiac tissues with minor expression in the kidney and gastrointestinal system[24]. Conversely, the β_2AR is predominantely expressed in the heart, lungs, skeletal muscle, liver and the uterus[24]. The β_3AR is predominately expressed in both brown- and white adipose tissue, the gastrointestinal tract and the prostate with minor expression in the heart[24]. The α_1ARs are expressed in the heart, liver, lung, prostate, spleen, kidney, brain and the aorta[25, 26]. Conversely, the α_2ARs have been detected in the brain, spleen, kidney, lung, prostate and heart[27]. The ubiquitous expression of the adrenergic receptors throughout the body clearly underscores the essential roles of these receptors in many diverse physiological processes.

Classical and non-traditional adrenergic signaling within the heart

Classical adrenergic signaling. Although nine subtypes of adrenergic receptors exist (β_1, β_2, β_3, α_1A, α_1B, α_1D, α_2A, α_2B, α_2C), human and murine hearts predominately express the β_1, β_2- and α_1 subtypes[28]. Adrenergic receptor subtypes modulate unique responses to catecholamine stimulation by activating different signaling pathways. Upon stimulation, the β_1AR couples to the Gs-protein which, in turn, activates a signaling cascade leading to cAMP

accumulation and protein kinase A (PKA) activation[28, 29]. In addition to Gs, β_2ARs are also known to uniquely couple to inhibitory G_i proteins, which inhibit adenylyl cyclase activation, thus reducing PKA activation[29-31]. Alternatively, stimulation of the α_1AR leads to both Gq and G_i coupling and subsequent phospholipase C (PLC) activation causing an increase in Ca^{2+} levels and protein kinase C (PKC) activation[28, 29, 32].

The adrenergic receptors modulate many physiological processes within the heart including chronotropy and inotropy as well as cardiomyocyte growth and survival (Fig 1.3). Acute activation of βARs represents the primary mechanism to increase cardiac performance under stress. Conversely, α_1 adrenergic activation is essential in cardiac myocyte survival and growth as well as cardiac fibroblast proliferation and fibrosis. Chronic stimulation of the ARs, however, has been implicated in numerous stages of cardiac remodeling including cardiomyocyte hypertrophy[33, 34] and apoptosis[30, 35, 36].

Both β_1 and β_2ARs are prototypical GPCRs expressed in animal hearts. βARs mediate increases in cardiac contraction upon agonist stimulation through the Gs-adenylyl cyclase-cAMP dependent protein kinase A (PKA) pathway[28, 29] (Fig 1.4). Conversely, β_2ARs are also known to uniquely couple to inhibitory Gi proteins leading to inhibition of adenylyl cyclases to reduce cardiac contraction, and initiation of anti-apoptotic and cell growth signaling[29, 37, 38] (Fig 1.4).

In addition to their roles in contractility, studies have implicated cardiac adrenergic receptors in the pathological remodeling leading to heart failure under chronic stimulation by elevated circulating plasma catecholamines and increased sympathetic tone. Evidence suggests distinct roles of the individual cardiac βAR and α_1AR subtypes in pathological hypertrophic remodeling. Chronic stimulation of the β_1AR is cytotoxic *in vitro*, leading to myocyte death and eccentric remodeling *in vivo*[28, 29, 35]. In heart failure, β_1AR expression is down regulated

although the mechanism, consequence and time point when this occurs are unknown[5]. Conversely, β_2AR signaling is cardioprotective and opposes β_1AR-induced cytotoxicity[28, 29, 35]. Alternatively, stimulation of the α_1AR signaling pathway induces hypertrophic signaling *in vitro*[30, 39] leading to concentric remodeling observed in compensatory hypertrophy *in vivo*. In particular, gene deficiency studies reveal α_1ARs as the major AR subtypes responsible for cardiac hypertrophy in animals[34, 40, 41]. Further, chronic stimulation of both the α_1AR[40, 41] and the β_1AR[42-46] are linked to catecholamine-induced cardiac remodeling *in vivo*. The α_1AR and βARs modulate hypertrophic signaling upon catecholamine stimulation by activating different signaling pathways. These pathways transduce their hypertrophic signal by activating members of the MAP kinase family, including the ERK1/2 (P44/P42) MAPK which translocate to the nucleus to activate transcription factors important in cellular differentiation, proliferation, and growth[47].

Non-traditional adrenergic signaling. Traditionally, the adrenergic receptors undergo conformational changes upon agonist stimulation that expose intracellular binding sites for the heterotrimeric G proteins within the third intracellular loop[19-21]. The recruited G proteins exchange GTP for GDP, leading to the dissociation of G proteins into activated α subunit and βγ dimers. The dissociated G proteins then trigger the activation of specific effector proteins (e.g. adenylate cyclase, phospholipase C). Second messengers, including cAMP, inositol trisphosphate and Ca^{2+}, are subsequently generated and induce a range of biological outcomes. Under the continued presence of agonist, the agonist-occupied receptor is phosphorylated on the cytoplasmic carboxyl terminus by G-protein receptor kinases (GRKs)[22, 23, 48, 49]. Once phosphorylated by GRKs, arrestin proteins are recruited to the receptor leading to receptor internalization via clathrin-coated pits[50]. This leads to receptor desensitization, a state in which

receptor no can no longer respond to further agonist stimulation (Fig 1.5). In addition to their roles in receptor desensitization and internalization, arrestin proteins have recently been shown to serve as adaptor proteins that facilitate the activation and subcellular localization of signaling cascades, including MAPK cascades[50-54] (Fig 1.6). Specifically, β arrestin 1 (also known as arrestin 2) and β arrestin 2 (also known as arrestin 3) facilitate ERK MAPK activation from both the β_1AR[55] and the β_2AR[51, 56]. This G protein-independent mechanism leads to cytoplasmic ERK retention and presumably phosphorylation of cytoplasmic targets, which modulate cellular function.

Adrenergic interactions in cardiac remodeling

βAR/α_1AR interactions. Despite extensive studies investigating the regulation of individual GPCR signaling cascades, the effect of concomitant GPCR activation by endogenous stimuli on downstream signaling remains poorly understood. There is a great deal of evidence supporting functional cross-talk between different GPCRs, such as between the β_1AR and the angiotensin 1 receptor[57], the α_2AR and the opioid receptors[58], as well as the c5a receptor and the UDP receptor[59] both *in vitro* and *in vivo*[57, 58, 60, 61]. The majority of these studies focus on short-term stress responses involved in modulation of common effectors such as G proteins, phospholipases, and adenylyl cyclases[61, 62]. Endogenous catecholamine stimulation activates both βAR and α_1AR signaling cascades, however, the concomitant activation of adrenergic signaling in the heart under chronic disease conditions is not known. Increasing evidence has implicated the importance of α_1AR and βAR interactions in the modulation of signaling pathways in the heart[29, 63-65]. These interactions occur either at the receptor level via physical interactions between the receptors, or downstream of the ARs via signaling pathway cross-talk.

It has been shown that α_1ARs and βARs form heterodimers[66, 67] and this interaction regulates receptor ontogeny, pharmacological diversity, signaling and regulation[68]. Alternatively, Gs stimulation inhibits Gq signaling via elevated regulator of G-protein signaling 2 (RGS2 levels[69]. Furthermore, Gq-induced PLC-β_1 activity is inhibited by Gs-induced PKA[70]. Together, these results suggest that βAR signaling may negatively regulate α_1AR signaling at the receptor level or down-stream.

βAR/IR interactions. In addition to adrenergic cross-talk, βARs have been shown to interact with receptor tyrosine kinases (RTKs) including the epidermal growth factor receptor[55, 71, 72], platelet-derived growth factor receptor[73, 74] and insulin-like growth factor receptor[75, 76]. Although the link between diabetes and heart failure has been established, the cellular mechanism remains unknown. Due to the high metabolic demand of cardiac cells during adrenergic stimulation, the interaction between βARs and the IR, within the heart represents a potentially essential mechanism leading to cardiac remodeling observed in diabetic patients. IRs and βARs induce heterologous signal transduction pathways leading to opposing cellular processes. IR, a member of the large RTK family, phosphorylates Insulin receptor substrate (IRS-1) leading to GLUT4 expression in effector cells. Increased GLUT4 expression on the cellular membrane induces glucose transport into the cell leading to anabolic processes. In addition, IRS-1 phosphorylation induces PI3K-mediated AKT activity leading to cardiac hypertrophy[77-79]. Conversely, ligand binding to the βARs, prototypical members of the G protein-coupled receptor superfamily, induces cAMP-dependent protein kinase A (PKA) activation and phosphorylation of Phospholamban (PLN) leading to increased Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) channel activity[28, 29, 80]. Elevated SERCA activity increases myocyte contractility, stroke volume and cardiac output[81].

Despite the different signaling mechanisms, insulin and adrenergic signaling is shown to interact to influence each other's signaling in adipocytes and liver cells. Two possible locations exist for IR/βAR cross-talk: the receptor level and down-stream signaling components. In adipocytes, IR activation leads to increased βAR down-regulation via phosphorylation of the β_2AR[76, 82-86]. This phosphorylation of the β_2AR by insulin-induced signaling pathways promotes insulin-induced β_2AR internalization in 3T3-L1 adipocytes and human epidermoid carcinoma A431 cells. Conversely, IGF-1 induces PI3K/Akt-dependent phosphorylation of β_1AR in CHO cells[87].

Meanwhile, other signaling proteins in GPCR signaling pathways also participate in RTK signaling cascades. In particular, the Gi protein is shown to be essential in glucose transport and insulin resistance in adipocytes and hepatocytes[88]. IRs have also been shown to recruit GRKs[91, 92] and the scaffold/adaptor protein β-arrestin[93-95]. Conversely, IGF-1-mediated MAP kinase (MAPK) phosphorylation is dependent on Gαi/βγ signaling[89, 90], or β arrestin 1 [96-98]. Further, down-regulation of β arrestin 1 by insulin treatment can impair MAP kinase signaling by both GPCRs and RTKs[99].

However, few studies have implied functional interactions between IRs and βARs in cardiac tissues. Insulin inhibits β-adrenergic action in the ischemic/reperfused heart to protect cardiac myocytes[100], and PI3K offsets cAMP-mediated positive inotropic effect via inhibiting calcium influx in cardiac myocytes[101]. In contrast, Akt activated by βAR/PKA signaling is involved in phosphorylation of the IR for insulin resistance in cardiac myocytes[102]. The mechanism and effects of decreased insulin signaling in response to βAR stimulation on cardiac remodeling remain largely unknown. Due to the high correlation between diabetes and cardiac remodeling as well as the intimate interactions between the βARs and IRs within the body, the

interactions between the βARs and the IRs within the heart represent a potentially essential mechanism to explain the link between diabetes and heart failure.

Clinical significance

Nearly sixty years after the discovery of the ARs, the 1960s witnessed not only the merger of biochemistry with pharmacology in the adrenergic receptor field, but also the utilization of βAR antagonists in clinical medicine[103]. Fifty years later, the cellular mechanisms of βAR blockade remain unknown. Clinically, βAR antagonists, including carvedilol, are effective long-term therapies for hypertension, cardiomyopathy and heart failure[104-110]. Although carvedilol has been used as an effective long-term therapy for heart failure, other drugs in the same class have been less successful or have failed in clinical trials[111]. In particular, the COMET trial showed that combined α_1AR and β_1AR antagonism via carvedilol treatment led to decreased mortality over metoprolol treatment, which blocks β_1AR alone[110]. In addition, insulin treatment increases the risk of mortality in HF patients[112]. Conversely, β blockade with either metoprolol[113] or carvedilol[114] results in significant mortality reduction compared to placebo in diabetic patients. These observations strongly suggest that a more defined understanding of the interactions between adrenergic receptors and other cardiac-relevant receptors is needed to improve the clinical utility of βAR antagonists in cardiovascular diseases, particularly heart failure. It is critical to understand the consequences of either activation or inhibition of adrenergic receptors in the hypertrophic growth of the heart. With this understanding, more efficient pharmacologic agents may be developed to prevent the progression of this major health problem.

Aims

Understanding how cells integrate information from a variety of chemically diverse signals into complex, orchestrated responses such as cell proliferation, differentiation and apoptosis is an overarching goal of cell biology. Adrenergic signaling within the heart represents essential signaling cascades regulating both cardiac performance as well as pathophysiological remodeling. Due to the complexities of hormonal signaling *in vivo,* it is essential to understand how concomitant activation of one signaling pathway can alter the physiologic outcome of another receptor's signaling. These studies not only underscore the critical role of signaling cross-talk in the complex regulation of receptor signaling via sub cellular localization of signaling components, but also provide novel mechanisms into the regulation of mitogenic signaling elicited by different receptors.

Broadly, with these following studies we hope to elucidate novel and physiologically relevant signaling cross-talk mechanisms and define their role in pathophysiological cardiac remodeling. An understanding of the mechanisms and physiologic consequences of adrenergic receptor cross-talk within the heart is essential to develop novel treatments designed to prevent cardiac remodeling and heart failure. The aims outlined below, which utilize a spectrum of physiological and cell biology techniques, will answer these basic questions and set a foundation for future areas of investigation.

Aim 1: To characterize the cellular mechanism by which GPCR cross-talk regulates mitogen-activated protein kinase (MAPK) activation and define how this regulation affects cellular proliferation.

Aim 2: To determine the mechanism by which type II RTK activation at high concentrations of mitogen recruits non-traditional GPCR signaling components to fine-tune activation of MAPK signaling and define how this regulation affects cellular proliferation.

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Figures & Figure Legends

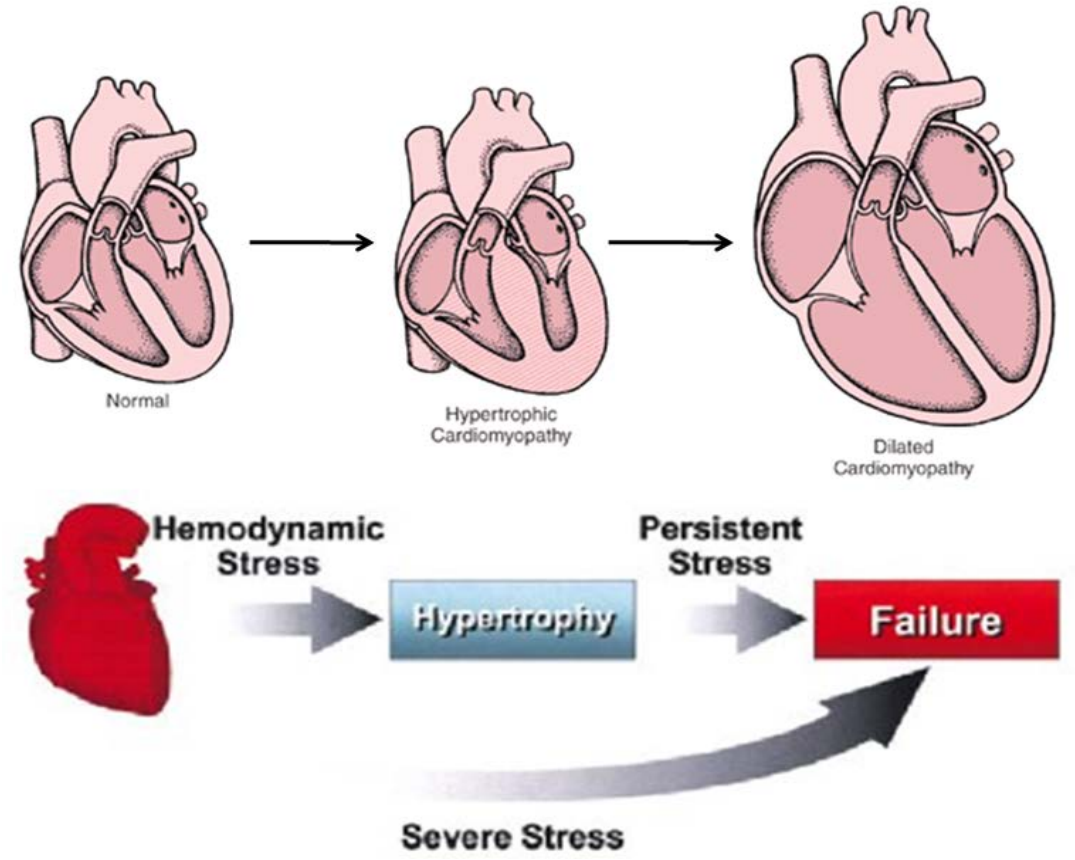

Figure 1.1 Stereotypical progression of cardiac remodeling leading to heart failure. Chronic hypertension (hemodynamic stress) elevates circulating blood catecholamines, leading to an initial compensatory cardiac hypertrophy (hypertrophic cardiomyopathy or concentric hypertrophy). Prolonged exposure to catecholamines induces myocyte apoptosis and fibroblast proliferation (dilated cardiomyopathy or eccentric hypertrophy). This weakens the left ventricular wall leading to eventual heart failure. Figure modified from www.ipmc.cnrs.fr and http://www.daviddarling.info/encyclopedia/C/cardiomyopathy.html

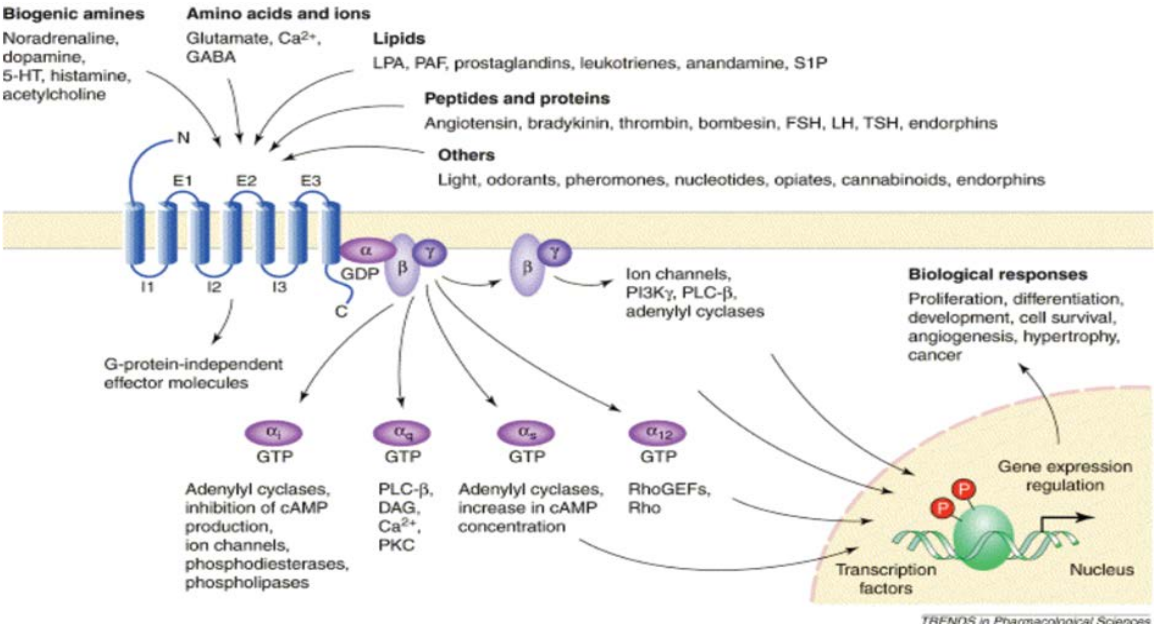

Figure 1.2. GPCR signaling pathways. GPCRs, including the adrenergic receptors, respond to a diverse variety of agonists, including both chemical and physical. Upon activation, conformational changes in the GPCR induce coupling to specific G proteins. This G protein coupling determines the consequence of GPCR activation by inducing distinct second messenger signaling pathways. Figure modified from http://isoft.postech.ac.kr/Research/POSBIOTM/content/intro.html

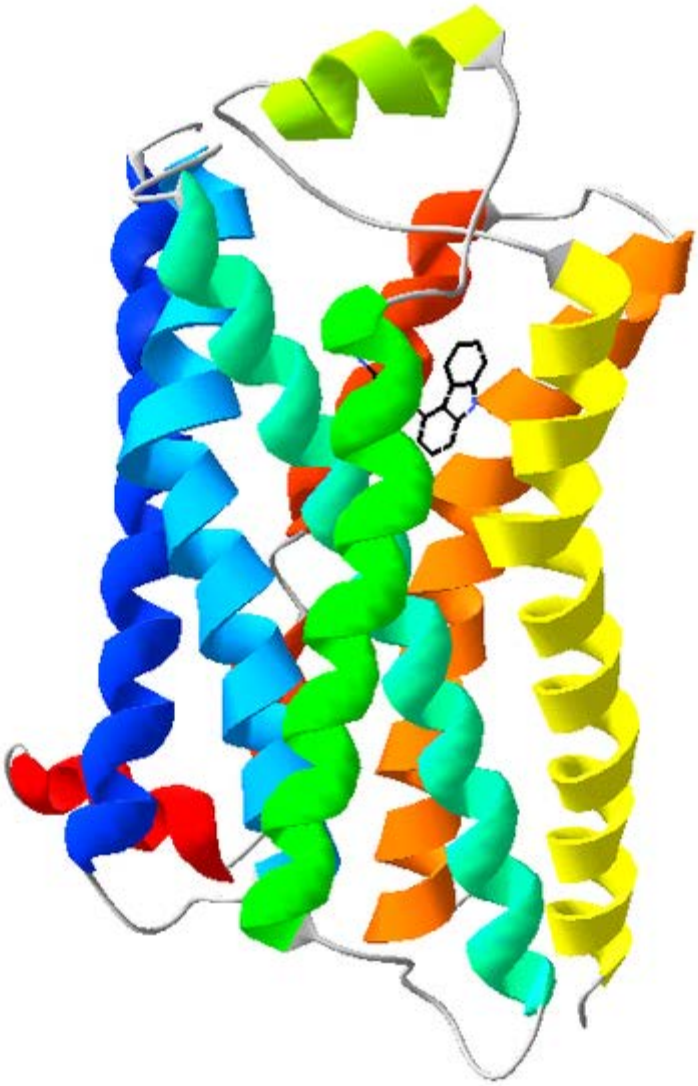

Figure 1.3. Crystal structure of the β_2 adrenergic receptor. The adrenergic receptors, prototypical G-protein coupled receptors, are comprised of seven transmembrane hydrophobic domains with an extracellular N terminus and a cytoplasmic C terminus. Here, the β_2AR is shown binding its inverse agonist carazolol. Figure obtained from Cherezov *et. al.* 2007 and Rosenbaum *et. al.* 2007[17, 18].

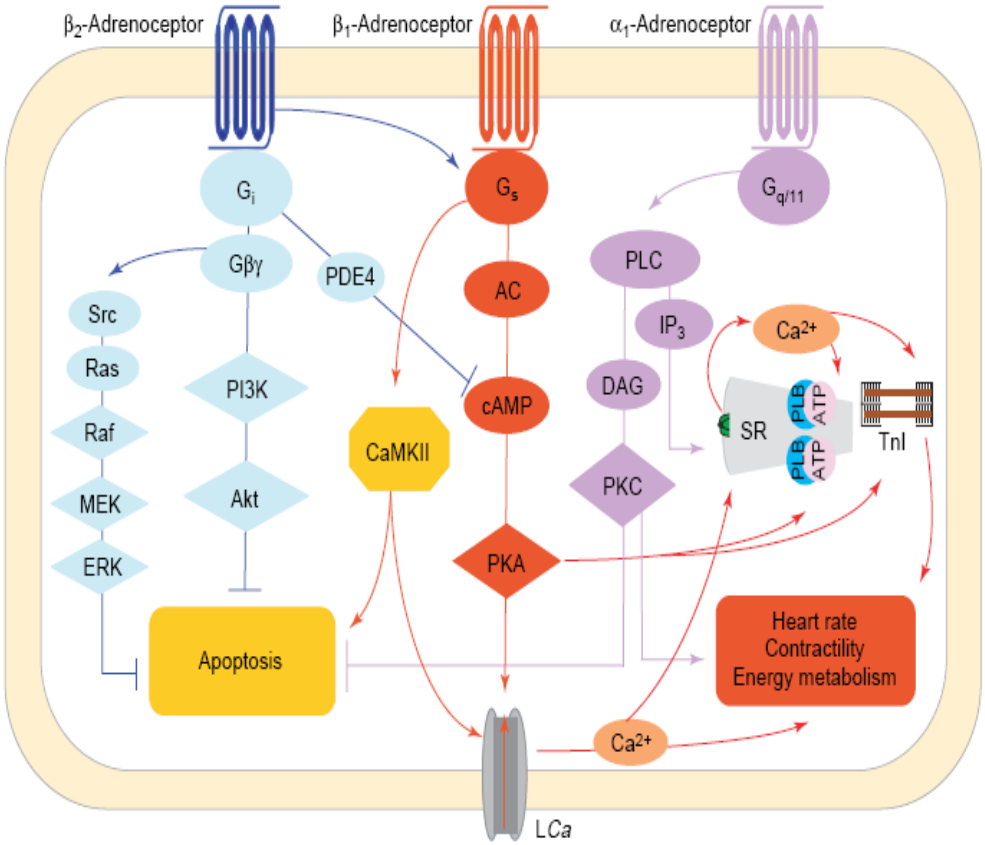

Figure 1.4. Adrenergic signaling in the heart. Within the heart, the β_1AR, β_2AR and α_1AR induce unique, but overlapping signaling pathways to modulate cardiac contraction, survival and growth. Figure obtained from Xiao *et. al.* 2006[29].

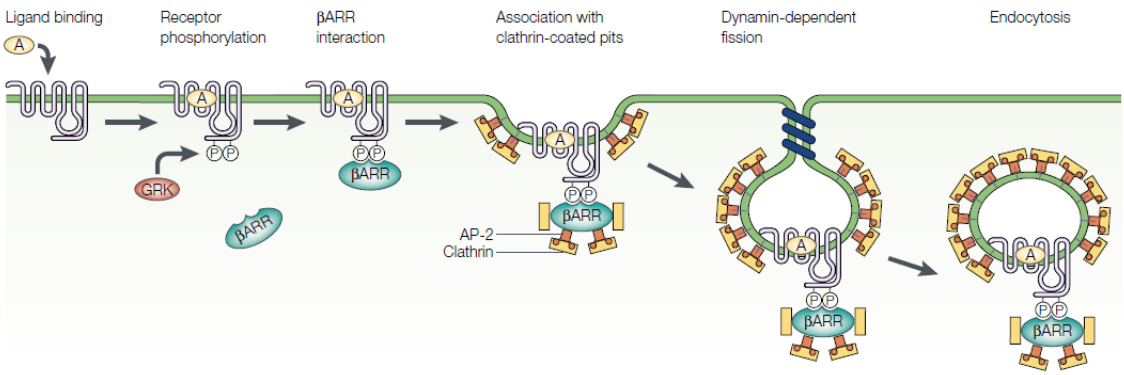

Figure 1.5. β_2AR desensitization upon agonist stimulation. Upon agonist binding, the β_2AR is phosphorylated on the cytoplasmic C-terminus by GPCR kinases (GRKs). This phosphorylation event recruits arrestin proteins to the receptor leading to receptor desensitization and internalization via clathrin-coated pits. Figure obtained from Pierce and Lefkowitz 2007[50].

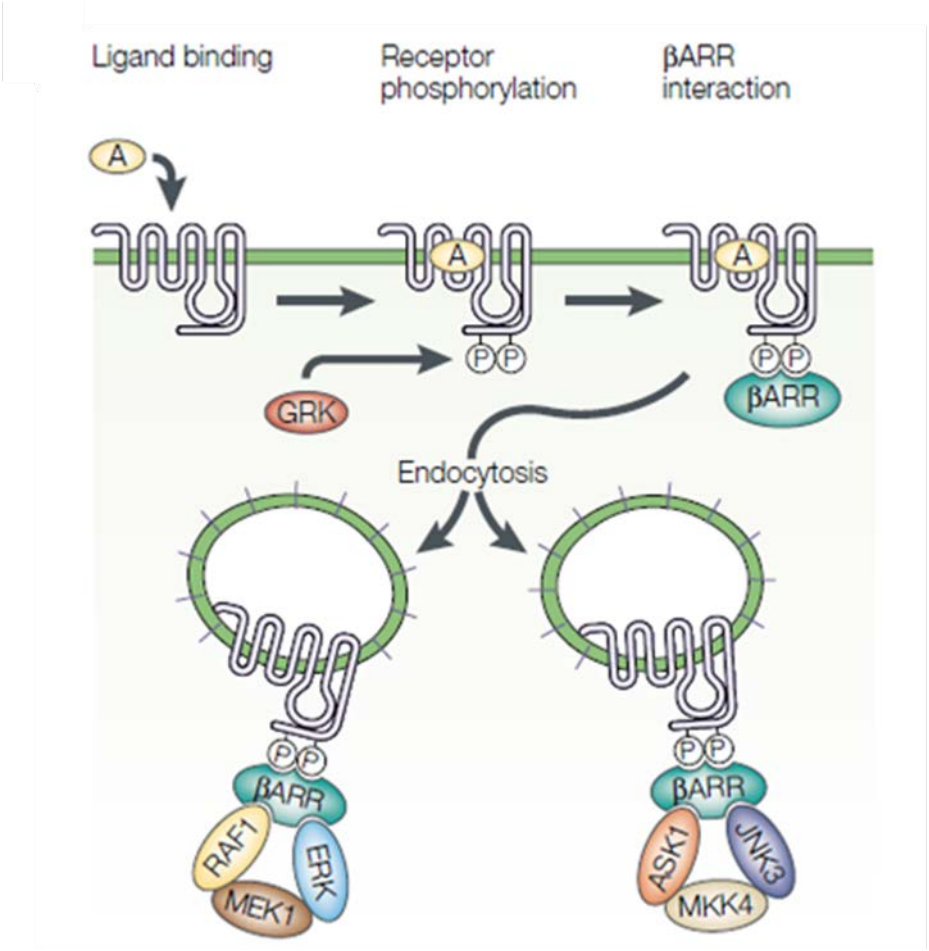

Figure 1.6. Arrestin-mediated scaffolding of MAPK signaling components. In addition to receptor internalization, arrestin proteins function as adaptor proteins to scaffold components of MAPK (ERK and JNK) signaling pathways. This offers a unique mechanism by which arrestin proteins can dictate the spatiotemporal activation of mitogenic signaling pathways. Figure obtained from Pierce and Lefkowitz 2007[50].

Chapter 2:

Arrestin orchestrates cross-talk between GPCRs to modulate the spatiotemporal activation of ERK MAPK

Abstract

G protein-coupled receptors (GPCRs) respond to diversified extracellular stimuli to modulate cellular function. Despite extensive studies investigating the regulation of single GPCR signaling cascades, the effects of concomitant GPCR activation on downstream signaling and cellular function remain unclear. We aimed to characterize the cellular mechanism by which GPCR cross-talk regulates mitogen-activated protein kinase (MAPK) activation. Adrenergic receptors on cardiac fibroblasts were manipulated to examine the role of arrestin in the spatiotemporal regulation of extracellular signal-regulated kinase (ERK)1/2 MAPK signaling. We show a general mechanism in which arrestin activation by one GPCR is capable of regulating signaling originating from another GPCR. Activation of Gq coupled-receptor signaling leads to prolonged ERK1/2 MAPK phosphorylation, nuclear accumulation, and cellular proliferation. Interestingly, coactivation of these receptors with the β-adrenergic receptors induced transient ERK signaling localized within the cytoplasm, which attenuated cell proliferation. Further studies revealed that recruitment of arrestin 3 to the β_2-adrenergic receptor orchestrates the sequestration of Gq-coupled receptor-induced ERK to the cytoplasm through direct binding of ERK to arrestin. This is the first evidence showing that arrestin 3 acts as a coordinator to integrate signals from multiple GPCRs. Our studies not only provide a novel mechanism explaining the integration of mitogenic signaling elicited by different GPCRs, but also underscore the critical role of signaling cross-talk among GPCRs *in vivo*.

Introduction

G protein-coupled receptors (GPCRs) respond to the most diversified extracellular stimuli to modulate cellular function. Activated receptors catalyze GDP/GTP exchange on cognate G proteins, which transduce downstream signals via second messengers and membrane channels [1]. Active GPCRs are phosphorylated by specific G-protein-coupled receptor kinases (GRKs) leading to receptor desensitization. Arrestin proteins, including arrestin 2 (also known as β arrestin 1) and arrestin 3 (also known as β arrestin 2) then bind to the phosphorylated receptor inducing clathrin-mediated internalization [2]. Despite extensive studies investigating the regulation of single GPCR signaling cascades, the effect of concomitant GPCR activation by endogenous stimuli on downstream signaling remains poorly understood. There is a great deal of evidence supporting functional cross-talk between different GPCRs both *in vitro* and *in vivo* [3-6]. The majority of these studies focus on short-term stress responses involved in modulation of common effectors such as G proteins, phospholipases, and adenylyl cyclases [5, 7]. However, chronic activation of multiple GPCR signaling pathways during adaptive tissue and organ remodeling suggests the potential of downstream cross-talk away from the plasma membrane.

One potential nexus for GPCR signaling cross-talk are the multi-functional scaffold proteins known as arrestins. Arrestins not only scaffold proteins necessary for the activation of different MAPK kinase families under single receptor activation, but also mediate transactivation of epidermal growth factor receptor signaling pathways [2, 8] and activation of many other non-GPCR signaling cascades [9]. In the case of GPCR-induced MAPK ERK activation, both G proteins and arrestins are capable of mediating ERK activation via independent mechanisms, with each pathway leading to unique spatiotemporal consequences [10]. While G-protein-activated ERK translocates to the nucleus for gene transcription, arrestins activate the ERK within the

cytoplasm. Since arrestins preferentially bind to some, but not all, GPCRs in a ligand-dependent manner, we envision that arrestins may play a role in GPCR cross-talk to coordinate MAPK activation in distinct subcellular compartments. Such a regulatory mechanism is essential for modulating MAPK signaling in divergent cellular functions such as cell proliferation and growth [11, 12], stress responses such as promoting cell mobility [13], and inhibiting cell apoptosis [14].

We choose cardiac fibroblasts as a model to study GPCR signaling cross-talk. Both α_1ARs and βARs are expressed in cardiac tissue and are activated by catecholamines to modulate cardiac remodeling including cardiac fibroblast proliferation by activation of distinct pathways. These pathways transduce signal by members of the MAP kinase family, including ERK1/2. Stimulation of the α_1AR leads to Gq coupling and subsequent phospholipase C (PLC) and protein kinase C (PKC) activation [15]. PKC has been shown to directly activate the Raf-MEK1-ERK axis. The activated ERK is translocated to the nucleus for gene transcription important in cellular differentiation, proliferation, and growth [16]. Meanwhile, stimulation of βAR signaling leads to ERK activation in a Gi-dependent manner via $G_{\beta\gamma}$ subunits [17]. Alternatively, activated βARs associate with arrestins leading to receptor internalization and arrestin-mediated ERK activation from both the β_1AR [18], and the β_2AR [10]. The later G protein-independent mechanism leads to cytoplasmic ERK retention and presumably phosphorylation of cytoplasmic targets.

Here we have identified a novel mechanism regulating Gq-coupled receptor-induced MAPK signaling via cross-talk with βAR-recruited arrestin in mouse cardiac fibroblasts and embryonic fibroblasts (MEFs). This is the first evidence suggesting that arrestin activation by one GPCR is capable of regulating a signaling pathway originating from another GPCR. These studies not only underscore the critical role of signaling cross-talk in the complex regulation of receptor signaling via subcellular distribution, but also provide a novel mechanism explaining

the coordination of subcellular mitogenic signaling elicited from different GPCR stimuli. Understanding regulatory cross-talk among GPCR signals will be critical for new pharmacological strategies aimed at preventing and treating the progression of a variety of diseases.

Materials & Methods

Cell culture, transfection, and adenoviral infections

Animal protocols are approved by the IACUC of the University of Illinois according to NIH regulation. Cardiac fibroblasts were isolated from the hearts of either wild-type, β_1KO or β_2KO neonatal FVB mice by a collagenase dispersion procedure utilizing a preplating step to remove cardiac myocytes as detailed in previous publications [1, 2]. Cells were cultured on 10 cm tissue culture treated dishes until 90% confluent. Cells were then plated on 35 mm tissue culture treated dishes at a confluency of 60-70%. Primary cardiac fibroblasts were maintained for 24 h in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and experiments were performed, unless otherwise noted, on cells serum-deprived for 24 h. Wild type, as well as arrestin 2 (also known as β arrestin 1) knock out (KO) , arrestin 3 (also known as β arrestin 2) KO and arrestin 2/3 double knock out (DKO) Mouse Embryonic Fibroblasts (MEFs) were maintained 24 h in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. FLAG-tagged mouse arrestin 3 constructs were transfected into primary neonatal cardiac fibroblasts utilizing Lipofectamine2000 (Invitrogen, Carlsbad, CA). Alternatively, neonatal cardiac fibroblasts were infected with recombinant adenoviruses as indicated after being cultured for 24 h. Recombinant plasmids expressing βARKct, GFP- arrestin 3, and GFP- arrestin 3 V54D have been generated as previously described 2, 4. GFP- arrestin 3 and GFP- arrestin 3 V54D

plasmids were gifts from Dr. Gang Pei (Chinese Academy of Sciences, Shanghai). Dominant negative Src plasmids were gifts from Dr. Qin Wang (University of Alabama at Birmingham). Mouse embryonic fibroblasts (MEF) cell lines were from Dr. Robert Lefkowitz (Duke University). GRK3ct is a gift from Dr. Blaxall Burns (University of Rochester). Co-immunoprecipitation experiments were conducted after 48 h expression.

Drug Treatment

Fibroblasts were treated with the following agonists or antagonists for the indicated times: epinephrine (Epi, 10 μM; Sigma, St. Louis, MO) or norepinephrine (NE, 10 μM; Sigma, St. Louis, MO) as general AR agonists, phenylephrine (Phe, 10 μM; Sigma, St. Louis, MO) as an α1AR agonist, isoproterenol (Iso, 10 μM; Sigma, St. Louis, MO) as a βAR agonist, clonidine (Clo, 10 μM; Sigma, St. Louis, MO) as an α2AR agonist, timolol (Tim, 10 μM; Sigma, St. Louis, MO) as a βAR antagonist, prazosin (Prz, 10 μM; Sigma, St. Louis, MO) as an α1AR antagonist, yohimbine (Yoh, 10 μM; Sigma, St. Louis, MO) as a α2AR antagonist, (Val5)angiotensin II (1μM; Sigma, St. Louis, MO) as an angiotensin agonist, thrombin (1nM; Sigma, St. Louis, MO) as a thrombin receptor agonist, phorbol 12-myristate 13-acetate (PMA, 10uM; Sigma, St. Louis, MO) as a PKC agonist. Fibroblasts were treated with the following inhibitors for the indicated times: persussis toxin (PTX, 500 ng/mL; Sigma, St. Louis, MO) as a Gi specific inhibitor, U73122 (1 μM; Calbiochem, San Diego, CA) as a non-specific phospholipase C (PLC) inhibitor, N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride; (H89, 10 μM; Sigma, St. Louis, MO) as a protein kinase A (PKA) inhibitor, or the membrane-permeable myristoylated PKC inhibitor fragment 19-27 (PKCi, 100 μM; MP Biomedicals, Irvine, CA).

Immunoblotting

Antibodies specific to both phosphorylated and total ERK1/2 were purchased from Cell Signaling (Danvers, MA). Fibroblasts were starved for 24 h prior to the addition of 10 μM of the indicated agonist or antagonist for the indicated times. Alternatively, fibroblasts were pretreated with inhibitors as indicated in the figure legends. After stimulation, fibroblasts were chilled, washed, and harvested in lysis buffer (5 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% NP-40, 2.5 mM Na4P2O7, 2.5 mM NaF, 0.5 mM PMSF, 40 μg ml-1 leupeptin and aprotinin). Lysates were clarified and resolved on 10% SDS-PAGE gels for western blot. In order to obtain nuclear extracts, stimulations were stopped on ice with PBS containing Ca2+ and Mg2+. Cells were scraped in hypotonic buffer (5mM NaCl, 20mM HEPES, 2.5mM PMSF, 2mM EDTA, 40μg/ml leupeptin and aprotinin, 10mM NaF, 1mM NaVO4, 0.4% NP-40) and disrupted by mechanical lysis. Nuclear extracts were pelleted by centrifugation at 12,000g for 1 min and resuspended in hypotonic buffer. Nuclear and cytoplasmic extracts were resolved via SDS PAGE. Signal intensity was quantitated by densitometry of Western blots. Phosphorylated and total ERK were detected via incubation with the corresponding antibodies (1:1000, Cell Signaling, MA) at 1:1000 for 2hr RT. Primary antibodies were visualized with IRDye 680CW goat-anti mouse or with IRDye 800CW goat-anti rabbit secondary antibodies using an Odyssey scanner (1:10,000; LI-COR Biosciences, Lincoln, NE). Phospho-ERK signals were corrected for total ERK levels and plotted as increase over basal levels.

Immunofluorescence microscopy

Fluorescent images were taken with a Zeiss Axioplan 2 microscope (Carl Zeiss Inc., Thornwood, NY) with an attached CCD camera and Metamorph software (Molecular Devices, Sunnyvale, CA). Cells were plated on laminin coated coverslips and then serum starved for 24 h. Fibroblasts were then stimulated with 10 μM of the indicated agonists or antagonists for the indicated times. Cells were then fixed with 4% paraformaldehyde for 1 hr at 4oC and then permeabilized with PBST (0.1% Triton X-100 in PBS) for 15 min at RT, rinsed and blocked, and then incubated with 1:400 HA antibody or 1:400 ERKp antibody diluted in PBST 2 hr at RT. AlexaFluor-594-conjugated goat anti-mouse IgG1 antibody or AlexaFluor- 488-conjugated goat anti-rabbit IgG1 were utilized as the secondary antibody at 1:1000 diluted in PBST with 10% FBS for 1 hr at RT.

Co-immunoprecipitation

Wild-type MEFs were transfected with FLAG-tagged mouse βAR together with either GFP-arrestin 3 or together with FLAG-tagged α_1A-AR, α_1B-AR, α_1D-AR, or β_2AR constructs as described above. MEFs were stimulated with 10 μM of the indicated agonist or antagonist for 10 min before being lysed with lysis buffer. Lysates were cleared by centrifugation and subjected to immunoprecipitation with M2 anti-FLAG affinity resin (25 μL, Sigma, St. Louis, MO). The immunoprecipitates were resolved via SDS-PAGE and blotted with antibodies against ERK (1:500, Cell Signaling, MA), FLAG (1:1,000; Sigma, St. Louis, MO), HA (1:1000, Covance, CA), GFP (1:1000, Rockland Immunochemicals, PA), or γ tubulin (1:5,000; Sigma, St. Louis, MO). Primary antibodies were visualized with IRDye 680CW goat anti mouse or with IRDye 800CW goat-anti rabbit secondary antibodies using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE).

Reverse Transcriptase PCR

RNA was extracted from neonatal cardiac fibroblasts according to protocol supplied with the Aurum total RNA minikit (Biorad, Hercules, CA). cDNA was created and amplified via PCR in a one step method using the protocol supplied with the Access RT-PCR system (Promega, Madison, WI). Briefly, 100ng RNA was amplified using 2mM MgSO4 utilizing the following thermocycler profile: reverse transcription at 45oC for 45 min, deactivation of reverse transcriptase at 94oC for 2 min, 45 cycles of denaturation at 94^{o}C for 1 min, annealing at 57oC for 1 min and extension at 68oC for 1 min. Primers against α1A-AR (forward: 5'-GTAGCCAAGAGAGAAAGCCG-3; reverse: 5'-CATCCCACCACAATGCCCAG-3') α1B-AR (forward: 5'-GCTCCTTCTACATCCCACTGG- 3'; reverse: 5'-AGGGGAGCCAACATAAGATGA-3') and α1D-AR (forward: 5'-CGTATGCTCCTTCTACCTCCC-3'; reverse: 5'-GCACAGGACGAAGACACCCAC-3') were designed based on previous publications 5, 6. PCR products were visualized on a 2% DNA gel.

Cardiac Fibroblast Cell Proliferation ELISA

The cell proliferation ELISA was carried out utilizing BrdU Labeling and Detection Kit III (Roche) according to the manufacturer's instructions. Briefly, cardiac fibroblasts were cultured in a 96-well plate as described above and left overnight to attach. 10 μM BrdU was added to cells prior to drug stimulation for 8 hours at 37^{o}C. Cells were then fixed and nucleases were allowed to partially digest cellular DNA. Cells were then incubated with an anti-BrdU antibody (200mU/ml) for 30 min at 37^{o}C. Colorimetric analysis was performed with a Spectramax M2 fluorometer reader. The data were normalized against the control.

GRK Knockdown

GRK2, GRK3 and GRK5 shRNA plasmids were obtained from Sigma. WT MEF cells were co-transfected with a GRK shRNA plasmid and a YFP plasmid for visualization. After overnight transfection, 10 μg/ml of puroymcin was added to dishes to select for positive transfection. After 48 hr puromycin selection, cells were serum starved for another 24 h. Cells were then either processed for imaging or harvested as described above for Western blot. Cell lysates were resolved via SDS-PAGE and blotted with antibodies against GRK2, GRK3 or GRK5 (SCBT,CA). Primary antibodies were visualized with IRDye 680CW goat-anti mouse or with IRDye 800CW goat-anti rabbit secondary antibodies using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE).

Statistical analysis

Data analysis was performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego CA).

Results

Agonist-dependent spatiotemporal activation of ERK upon stimulation of adrenergic receptors in cardiac fibroblasts

Both α_1ARs and βARs are implicated in promoting ERK activation upon stimulation with catecholamines [12]. Utilizing cardiac fibroblasts, we investigated the mechanism leading to the modulation of ERK signaling upon potential cross-talk between these GPCRs. We examined agonist-dependent ERK phosphorylation induced by epinephrine (Epi). Since α_1ARs are the primary adrenergic receptors responsible for ERK activation in cardiac tissues, we used the α_1AR-specific agonist phenylephrine (Phe). Both Epi and Phe activated ERK by increasing the

phosphorylation of ERK (phospho-ERK). The maximal phospho-ERK levels peaked at 5 minutes after stimulation, with the peak levels induced by Phe significantly lower than that by Epi. Moreover, the Epi-induced phospho-ERK underwent a rapid decrease to baseline, whereas, the Phe-induced phospho-ERK signal was prolonged and returned to baseline levels slowly (Fig 2.1A).

Previous studies have indicated that subcellular ERK distribution may shape the temporal profile of ERK activation [18]. We found that Epi-induced phospho-ERK accumulated in the cytoplasm and returned to baseline levels rapidly (Fig 2.1B). Interestingly, Phe-induced phospho-ERK translocated to the nucleus and remained elevated throughout stimulation (Fig 2.1B). The subcellular distribution of phospho-ERK was confirmed in fractionation studies. The Epi-induced phospho-ERK was enriched in the cytoplasmic fraction; however, the Phe-induced phospho-ERK was enhanced in both the cytosolic and nuclear fractions (Fig 2.1C). Thus, concomitant activation of the β and α_1ARs by Epi induces a distinct spatiotemporal ERK signal than that elicited by activation of the α_1AR alone by Phe.

ERK activation is induced by a classical α_1AR/Gq pathway in cardiac fibroblasts

To investigate the mechanism underlying the modulation of ERK signaling upon adrenergic receptor cross-talk, we first examined which adrenergic receptor was responsible for ERK phosphorylation. Stimulation with Epi and Phe induced potent ERK activation (Fig 2.2A). Stimulation with the βAR agonist isoproterenol (Iso) resulted in minimal ERK activation, whereas, stimulation of the α_2ARs with clonidine (Clo) did not activate ERK (Fig 2.2A). In addition, pretreatment with the α_1AR antagonist prazosin (Prz), but not the βAR antagonist timolol (Tim) or the α_2AR antagonist yohimbine (Yoh), significantly blocked Epi-induced ERK activation (Fig 2.2B and data not shown). As a control, Prz and Yoh, as well as a panel of β-

blockers including Tim, propanolol, alprenolol, and cavedilol did not alter basal ERK levels (Supplementary Figure 2.I). Previous studies show that activation of βARs can induce cell-specific ERK activation [19]. Similar to neonatal cardiac fibroblasts, Iso induced minimal ERK phosphorylation in MEFs, and in both neonatal and adult cardiac myocytes. In contrast, stimulation of βARs with Iso induced robust ERK phosphorylation in HEK293 cells (Supplementary Figure 2.II). Together, these results confirmed that ERK activation resulted primarily from α_1AR stimulation in cardiac fibroblasts, which was supported by the expression of both α_{1A}AR and α_{1B}AR genes (Supplementary Figure 2.III). Accordingly, stimulation of Gq with *Pasteurella multocida* toxin and stimulation of PKC with phorbol myristate acetate (PMA) was sufficient to induce potent ERK activation (Fig 2.2 and data not shown). In contrast, direct inhibition of Gi with *Pertussis* toxin (PTX) had no effect on agonist-induced ERK activation (Supplementary Figure 2.IV). Pretreatment with the PKA inhibitor H89, which blocks Gs signaling, also had no significant effect on agonist-induced ERK activation (Supplementary Figure 2.IV). Together, these data confirm that the α_1ARs are the primary adrenergic subtypes responsible for ERK phosphorylation via Gq activation in cardiac fibroblasts.

Classic α_1AR/Gq coupling activates phospholipase C to produce diacyl glycerol, leading to PKC activation. Pretreatment with the PLC inhibitor U73122 significantly blocked both Epi- and Phe-induced ERK phosphorylation (Figs 2.2C and 2.2D). Similarly, pretreatment with a myristolated PKC inhibitory peptide (PKCi) significantly prevented ERK activation upon either Epi or Phe stimulation (Figs 2.2C and 2.2D). Moreover, inhibition of MEK with U1026 also prevented both Epi and Phe-induced ERK activation (Figs 2.2C and 2.2D). These data confirm that α_1AR-induced ERK signaling is dependent on activation of PLC and PKC leading to the Raf-MEK pathway [20].

βAR activation prevents nuclear translocation of α_1AR-induced ERK activation and cardiac fibroblast proliferation

One possible explanation for the differences in the spatiotemporal ERK activation profile between Epi and Phe is that βAR activation by Epi may lead to cytoplasmic retention of α_1AR-induced phospho-ERK. To test this hypothesis, we blocked the βARs with Tim before Epi stimulation. Inhibition of the βARs with Tim redistributed the Epi-induced phospho-ERK to both the cytoplasm and the nucleus, resulting in a similar spatial profile to that induced by Phe (Figs 2.3A and 2.3B). As a result, βAR blockade significantly prolonged the phospho-ERK signal (Fig 2.3C). Moreover, simultaneous stimulation of βARs with Iso prevented nuclear translocation of Phe-induced phospho-ERK and promoted phospho-ERK signal attenuation (Supplemental Figure 2.V). Together, our data suggest that activation of βARs can prevent nuclear translocation of phospho-ERK induced by the α_1AR/Gq signaling pathway.

To understand the physiologic consequence of cytoplasmic ERK sequestration upon α_1AR and βAR cross-talk in cardiac fibroblasts, we examined cell proliferation utilizing BrdU incorporation upon different ligand stimulation. Activation of the α_1ARs with Phe, but not the βARs with Iso, induced a significant increase in BrdU incorporation, which was blocked by the MEK inhibitor U1026. Further, activation of both the α_1AR and βARs with Epi did not significantly enhance BrdU incorporation. However, inhibition of βARs with Tim enabled Epi to stimulate BrdU incorporation (Fig 2.3D), presumably due to the redistribution of phospho-ERK to the nucleus for gene transcription (Fig 2.3A). This increase in BrdU incorporation was again blocked by U1026.

β_2AR, but not β_1AR, activation prevents nuclear translocation of α_1AR-induced ERK activation

We then sought to identify the βAR subtype responsible for cytoplasmic sequestration of α_1AR-induced ERK. Cardiac fibroblasts lacking the β_2AR, but not those lacking the β_1AR, displayed nuclear phospho-ERK accumulation upon Epi stimulation (Figs 2.4A and 2.4B), which also prolonged the phospho-ERK signal (Fig 2.4C). Stimulation of DKO cells, which lack both βAR subtypes, with Epi induced phospho-ERK in both the cytoplasm and nucleus (Figs 2.4D and 2.4E). We then reintroduced either β_1AR or β_2AR into DKO cells with similar expression levels (data not shown). Expression of β_2AR, but not β_1AR, recovered the cytoplasmic retention of phospho-ERK induced by Epi (Figs 2.4D and 2.4E). Thus, the effect is β_2AR-specific, and is not due to the higher endogenous expression levels of the β_2AR than the β_1AR in cardiac fibroblasts [21]. Together, these data suggest that activation of the β_2AR modulates both the spatial and temporal profile of α_1AR-induced ERK activation in cardiac fibroblasts.

β_2AR-dependent recruitment of arrestin is necessary to sequester α_1AR-induced ERK signaling

We further examined the molecular mechanism explaining this signaling cross-talk. Previous studies reported that α_{1D}ARs form a heterodimer with β_2ARs in HEK293 cells, thus altering α_{1D} signaling [22], but this α_1AR subtype is not expressed in cardiac fibroblasts (Supplementary Figure 2.III). Moreover, neither α_{1A}AR nor α_{1B}AR dimerized with β_2AR (Supplementary Figure 2.VI). Alternatively, upon phosphorylation of the β_2ARs via GRKs, arrestins are recruited leading to receptor internalization [23]; and the internalized β_2AR/arrestin complexes propagate numerous signaling pathways, including ERK pathways [24]. Due to their role in arrestin recruitment, we investigated the role of GRKs in Epi-induced ERK activation.

MEF cells were used for selective knockdown of individual GRKs. In wild-type MEF cells, Epi stimulated cytoplasmic phospho-ERK, similar to cardiac fibroblasts. Selective knockdown of GRK2, but not other GRKs, in MEF cells significantly promoted nuclear accumulation of phospho-ERK induced by Epi (Fig 2.5A and Supplementary Figure 2.VII). Expression of βARKct, a GRK2 inhibitor that prevents arrestin recruitment and thus βAR internalization [25], significantly promoted nuclear accumulation of phospho-ERK (Fig 2.5B) and prolonged ERK signaling (Fig 2.5C). These data indicate that the GRK2-mediated phosphorylation of the β_2AR modulates ERK activation, presumably through arrestin-mediated scaffolding of ERK.

In addition, Src is necessary for arrestin-dependent βAR internalization upon agonist binding [26]. Inhibition of Src with either over-expression of dominant negative Src (DN-Src) or treatment with Src inhibitor PP2 prolonged ERK activation induced by Epi, but had no effect on Phe-induced ERK (Supplementary Figure 2.VIII and data not shown). Fractionation studies revealed that DN-Src expression enhanced nuclear phospho-ERK translocation upon Epi stimulation (Supplementary Figure 2.VIII). These data suggest that Src-dependent and arrestin-mediated β_2AR internalization is necessary for cytoplasmic sequestration of the α_1AR-induced ERK signal under Epi stimulation.

To identify the arrestin(s) responsible for this cross-talk, MEF cells lacking either arrestin 2 (also known as β arrestin 1) or arrestin 3 (also known as β arrestin 2) were used. In comparison to wild-type MEF cells, Epi promoted phospho-ERK translocation to the nucleus in arrestin 3 KO (arr3-KO), but not arrestin 2 (arr2-KO) MEF cells (Figs 2.6A and 2.6B). Thus, arrestin 3 is primarily responsible for the cross-talk between the β_2 and α_1ARs. We then examined the association between ERK and the β_2AR/arrestin 3 complex upon agonist stimulation. Epi induced a significant increase in the association between ERK and the

β_2AR/arrestin 3 complex, which was attenuated by Tim (Fig 2.6C and Supplementary Figure 2.IX). In contrast, neither Phe nor Iso enhanced the association between ERK and the β_2AR/arrestin 3 complex; but co-stimulation with Iso and Phe promoted formation of the complex (Fig 2.6C). Further, a dominant-negative arrestin 3 (GFP-V54Darr3 [27]) also formed a complex with the β_2AR, but was not sufficient to promote the binding of ERK to the β_2AR/arrestin 3 complex (Fig 2.6C). We then used GFP-V54Darr3 to further perturb the cross-talk between the β_2 and α_1ARs. Expression of GFP-V54Darr3, but not GFP-arr3, promoted nuclear translocation of the phospho-ERK induced by Epi, which did not colocalize with GFP-V54Darr3 in the cytoplasm (Figs 2.6D and 2.6E). In contrast, expression of neither GFP-arr3 nor GFP-V54Darr3 affected the Phe-induced nuclear accumulation of phospho-ERK (Figs 2.6D and 2.6E). In cells expressing GFP-V54Darr3, the cytosolic and the nuclear accumulation of Epi-induced phospho-ERK was further confirmed by fractionation studies (Fig 2.6F). Not surprisingly, nuclear translocation also prolonged the Epi-induced phospho-ERK signal (Fig 2.6G). Together, these data show that internalization of the β_2AR via arrestin 3 induces the cross-talk between the α_1 and β_2ARs, leading to ERK sequestration within the cytoplasm.

Arrestin modulation of cellular ERK signaling via GPCR cross-talk is a general mechanism

To test whether this arrestin-mediated GPCR cross-talk is a general MAPK regulatory mechanism upon concomitant activation of multiple GPCRs, we used MEF cells lacking both arrestin 2 (also known as β arrestin 1) and arrestin 3 (also known as β arrestin 2) to investigate phospho-ERK distribution induced by different Gq-coupled receptors in the absence and presence of β_2AR activation. Stimulating both wild type MEFs and cardiac fibroblasts with either Phe or PMA induced nuclear accumulation of phospho-ERK. Interestingly, stimulation of wild type MEF and cardiac fibroblast cells with two other Gq-coupled receptor agonists ((Val5)

angiotensin II or thrombin) also induced nuclear phospho-ERK accumulation (Figs 2.7A and 2.7B), likely through Gq-dependent pathways [28]. However, upon Iso co-stimulation, Phe-, (Val5) angiotensin II-, thrombin-, as well as PMA-induced phospho-ERK was sequestered within the cytoplasm in both wild type MEF and cardiac fibroblast cells (Figs 2.7A and 2.7B). As expected, in MEF cells lacking arrrestins, or in cardiac fibroblasts expressing the GFP-V54Darr3 mutant, ERK signaling induced by the different stimuli was able to translocate into the nucleus. However, both arrestin deficiency and GFP-V54Darr3 expression blocked the effect of β_2AR activation under Iso stimulation (Figs 2.7A and 2.7B). Together, these data suggest that arrestin activation by the β_2AR can sequester Gq-coupled receptor-induced phospho-ERK within the cytoplasm in both cardiac fibroblasts and MEF cells.

Discussion

In this study, we have identified a novel mechanism regulating Gq-coupled receptor-induced ERK MAPK signaling via cross-talk with β_2AR-recruited arrestin 3 in cardiac fibroblasts and MEFs (Fig 2.8). This is the first evidence suggesting that arrestin activation by one receptor is capable of regulating signaling originating from another GPCR. G protein-independent regulation of GPCR signaling via arrestins is an emerging mechanism explaining the regulation of a growing list of GPCR-mediated signaling including α_2AR [18], angiotensin receptors [24], β_1AR [17], β_2AR [10], opioid receptors [29] and the vasopressin receptors [30, 31]. Recruitment of arrestins to a phosphorylated GPCR regulates not only receptor internalization, but also intracellular signaling such as transactivation of receptor tyrosine kinases [2, 8]. Arrestin, in addition, scaffolds ERKs leading to activation, cytoplasmic retention [24, 32], and decreased transcription in the nucleus [33]. In reconstituted systems this provides a linear mechanism,

however, this model fails to reflect the convoluted signaling networks *in vivo*. Endogenous ligands, including neurotransmitters and hormonal peptides, bind to multiple receptors present in a cell, activating numerous signaling cascades. Indeed, accumulative evidence supports signal cross-talk among GPCRs, such as between the β_1AR and the angiotensin 1 receptor [6], the α_2AR and the opioid receptors [4], as well as the c5a receptor and the UDP receptor [34]. Here, our results suggest that arrestin functions as a master regulator, coordinating subcellular ERK activation under multiple extracellular stimuli to inhibit nuclear translocation and facilitate signal attenuation. Considering the ability of arrestin to scaffold different cytoplasmic signaling components besides ERK, such as Src, JNK, and p38 [28], and the ability of arrestin to selectively bind to some, but not all GPCRs in an agonist-dependent fashion [35], our data suggest a general mechanism of arrestin-mediated cross-talk among GPCRs with broad implications in physiological responses under neurohormonal regulation *in vivo*.

In cardiac fibroblasts and MEFs, our data indicate that the α_1ARs make the primary contribution to ERK activation, supporting the dominant roles of these receptor subtypes in cardiac remodeling [36]. Consistent with previous studies [37], stimulation of the α_1ARs with Phe induces the classic Gq-dependent activation of PLC and PKC, leading to ERK activation through Raf-MEK1 kinase cascade. Under this signaling cascade, activated ERK translocates to the nucleus [37]. Interestingly, this scenario is completely reshaped when β_2ARs are co-activated with the α_1AR upon Epi stimulation. Co-activation of the α_1 and β_2ARs leads to sequestration of phospho-ERK within the cytoplasm. Under Epi stimulation, it was possible that two pools of ERK existed; a transient pool activated by the β_2AR and a prolonged pool activated by the α_1AR. The first pool becomes dominant simply because β_2ARs are more prominent in cardiac tissues than α_1ARs. This explanation is unlikely for several reasons. First, α_1AR antagonist prazosin

blocked Epi-induced ERK phosphorylation. Second, stimulation with isoproterenol (a βAR agonist) alone induces minimal ERK activation. Third, blockade of the β_2AR prolonged ERK activation and only slightly decreased maximal ERK levels (Fig 2.3C). Taken together, these data support that ERK activation by Epi and Phe originates from α_1AR activation. However, under Epi stimulation, β_2AR activation changes the spatiotemporal profile of ERK signaling by policing ERK to the β_2AR/arrestin 3 complex. Cytoplasmic retention may allow targeting to non-nuclear ERK substrates involved in different cellular stress processes [38] and receptor desensitization [39]. This unique observation highlights the potential diversified physiological consequences under a given extracellular environment when multiple receptors are activated simultaneously. As expected, this arrestin 3-mediated cross-talk is dependent on the receptor phosphorylation by GRK. Inhibiting GRK2, but not other GRKs, is sufficient to prevent the β_2AR-dependent modulation of α_1AR-induced ERK signaling. In addition to sequestering ERK within proximity of cytoplasmic ERK targets, our data indicate that interaction with arrestin 3 may facilitate the attenuation of ERK signaling in the cytoplasm [40]. However, inhibition of phosphatase 2A or dual-specificity phosphatases (DUSPs) failed to prevent ERK dephosphorylation (data not shown), thus the phosphatases involved remain to be identified.

The distinct spatiotemporal profile of ERK activation induced by Gq-coupled receptors in the presence or absence of β_2AR (and potentially other GPCRs) activation has broad implications in the maladaptative remodeling of different tissues *in vivo*. Indeed, in cardiac myocytes, the β_1AR, but not the β_2AR appears to cross-talk with Gq-coupled receptors in modulating ERK activation (unpublished data). Gq-coupled receptor signaling and ERK activation play essential roles in long-term pathophysiological cardiac remodeling [11, 36, 41]. Traditionally, activated ERK MAPK translocates to the nucleus to activate transcription factors including Elk-1 [42] and GATA-

4 [43] leading to fibroblast proliferation and myocyte hypertrophy. Here, this nuclear translocation of ERK is abrogated by arrestin binding to activated β_2ARs in cardiac fibroblasts; interestingly it can be restored with the clinically-relevant β blocker timolol. Our data thus provide insights into understanding the tissue remodeling observed in patients under chronic treatment with β-blockers in various clinical and physiological conditions.

In summary, our findings provide the first evidence for the role of β_2AR-recruited arrestin in regulating signaling from another GPCR. These findings provide a novel mechanism to significantly advance our understanding of the growing profiles of GPCR regulatory pathways. These data further underscore the critical role of signaling cross-talk in the complex regulation of receptor signaling via subcellular localization of signaling components, which will have significant implications in numerous clinical and physiological conditions.

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Figures & Figure Legends

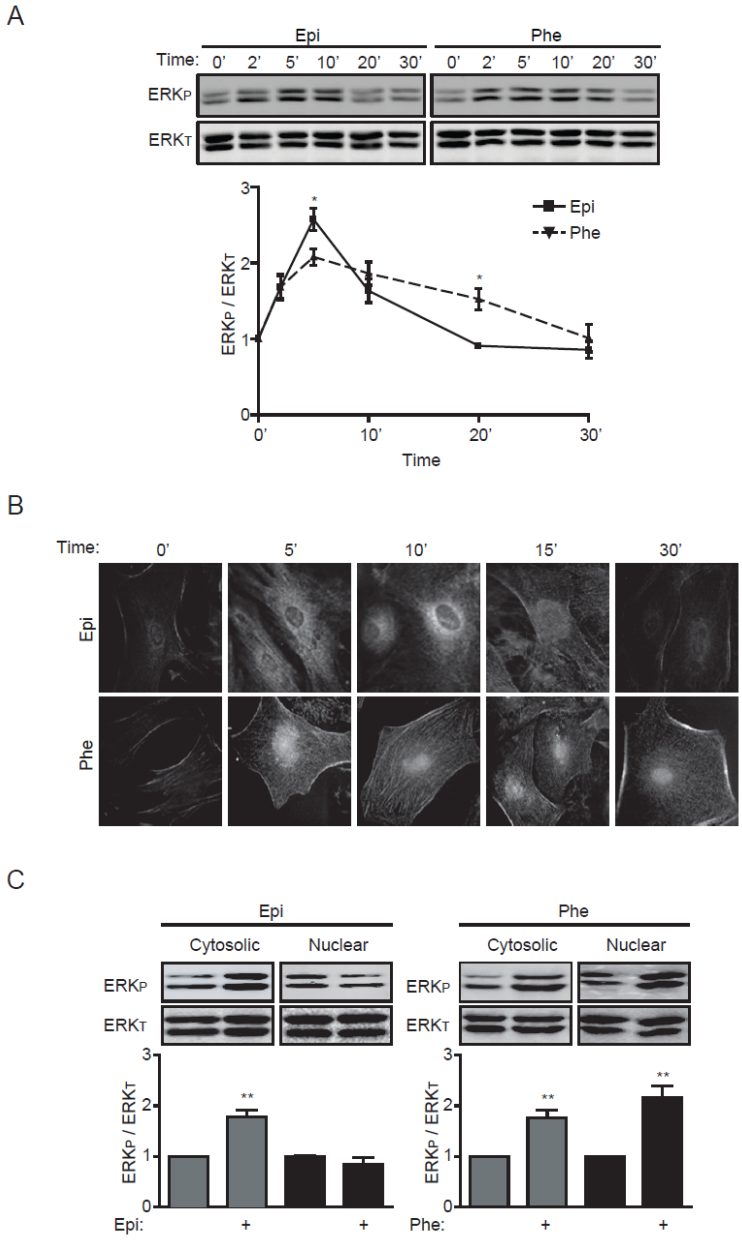


Figure 2.1. The temporal and spatial patterns of ERK activation are agonist-dependent. A, Epi (10 μM) and Phe (10 μM) induced distinct temporal patterns of ERK phosphorylation in wild type (WT) cardiac fibroblasts. B, Phosphorylated ERK was detected by immunofluorescence staining. Epi (10 μM) induced transient cytoplasmic ERK phosphorylation whereas Phe (10 μM) induced prolonged nuclear ERK phosphorylation. C, WT cardiac fibroblasts were treated with either Epi (10 μM) or Phe (10 μM) before being lysed and separated into nuclear and cytosolic fractions. Epi induced transient cytoplasmic ERK phosphorylation whereas Phe induced prolonged cytoplasmic and nuclear ERK phosphorylation. Phospho-ERK (ERKp) was normalized against total ERK_T. N=4; *, $p<0.05$; **, $p<0.01$ by unpaired student *t*-test.

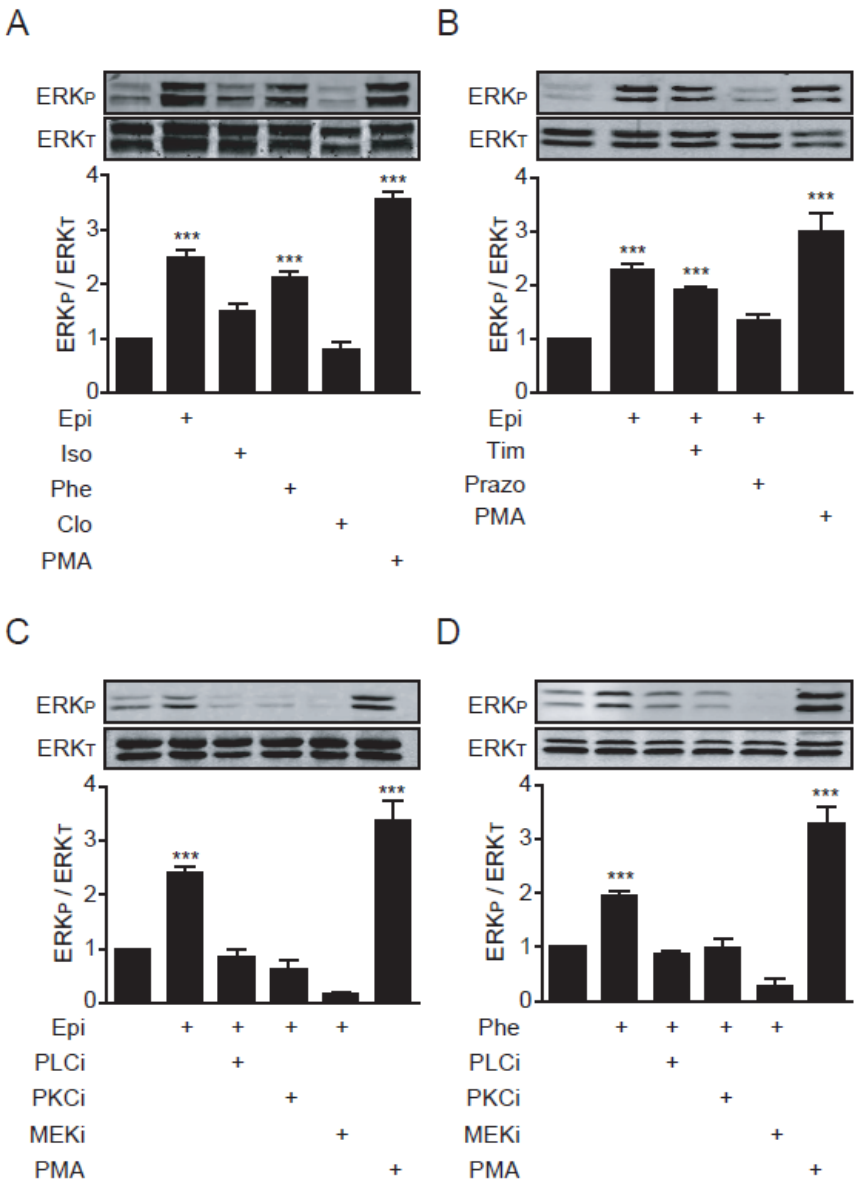


Figure 2.2. ERK activation upon adrenergic stimulation occurs via α_1AR signaling. A, WT cardiac fibroblasts were stimulated with 10 μM of either Epinephrine (Epi), Isoproterenol (Iso), Phenylephrine (Phe), Clonidine (Clo) for 5 mins or PMA for 10 mins to obtain maximal stimulation. B, WT fibroblasts were pretreated with 10 μM of either prazosin (Prz) or timolol (Tim) for 5 mins. Cells were then stimulated with 10 μM of either Epi or Phe for 5 mins or PMA for 10 mins. C and D, WT fibroblasts were pretreated for 30 mins with the PLC inhibitor U73122 (1 μM), PKC inhibitor PKCi (20 μM), or a MEK inhibitor (1 μM), and then stimulated with either Epi or Phe for 5 minutes. Phospho-ERK (ERKp) was normalized against total ERK_T. N=3; ***, $p<0.001$ by unpaired student *t*-test.

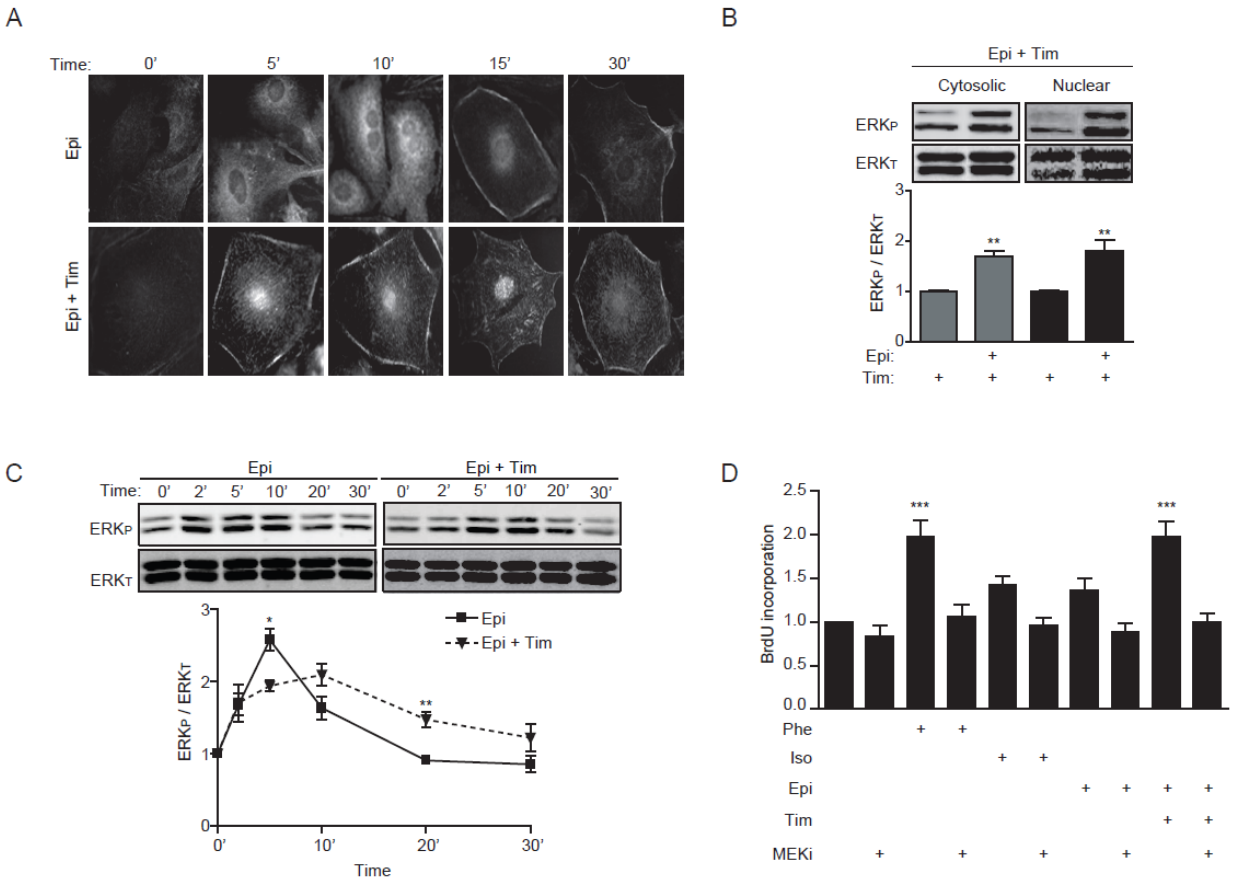


Figure 2.3. Inhibition of βAR activation permits nuclear localization of α_1AR-induced ERK signal and stimulates cell proliferation. A, WT cardiac fibroblasts were pretreated, if indicated, with the βAR antagonist Tim (10 μM) for 5 min and then stimulated with Epi (10 μM). Cells were fixed for phospho-ERK (ERKp) staining. While Epi-induced ERKp signal was localized in the cytoplasm, Tim pretreatment relocated Epi-induced ERKp signal to the nucleus. B, WT fibroblasts were pretreated with Tim before Epi stimulation, the cells were then lysed and separated into nuclear and cytosolic fractions. C, WT fibroblasts were pretreated with or without Tim for 5 minutes and then stimulated with Epi for the indicated times. D. WT fibroblasts were pretreated with either Tim (10 μM) or the MEK inhibitor (1 μM) for 30 minutes before stimulation with Iso (10 μM), Phe (10 μM), or Epi (10 μM) for 8 hours. BrdU incorporation was measured, and the data were normalized against the control. Phospho-ERK (ERKp) was normalized against total ERK_T. N=3; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ by unpaired student *t*-test.

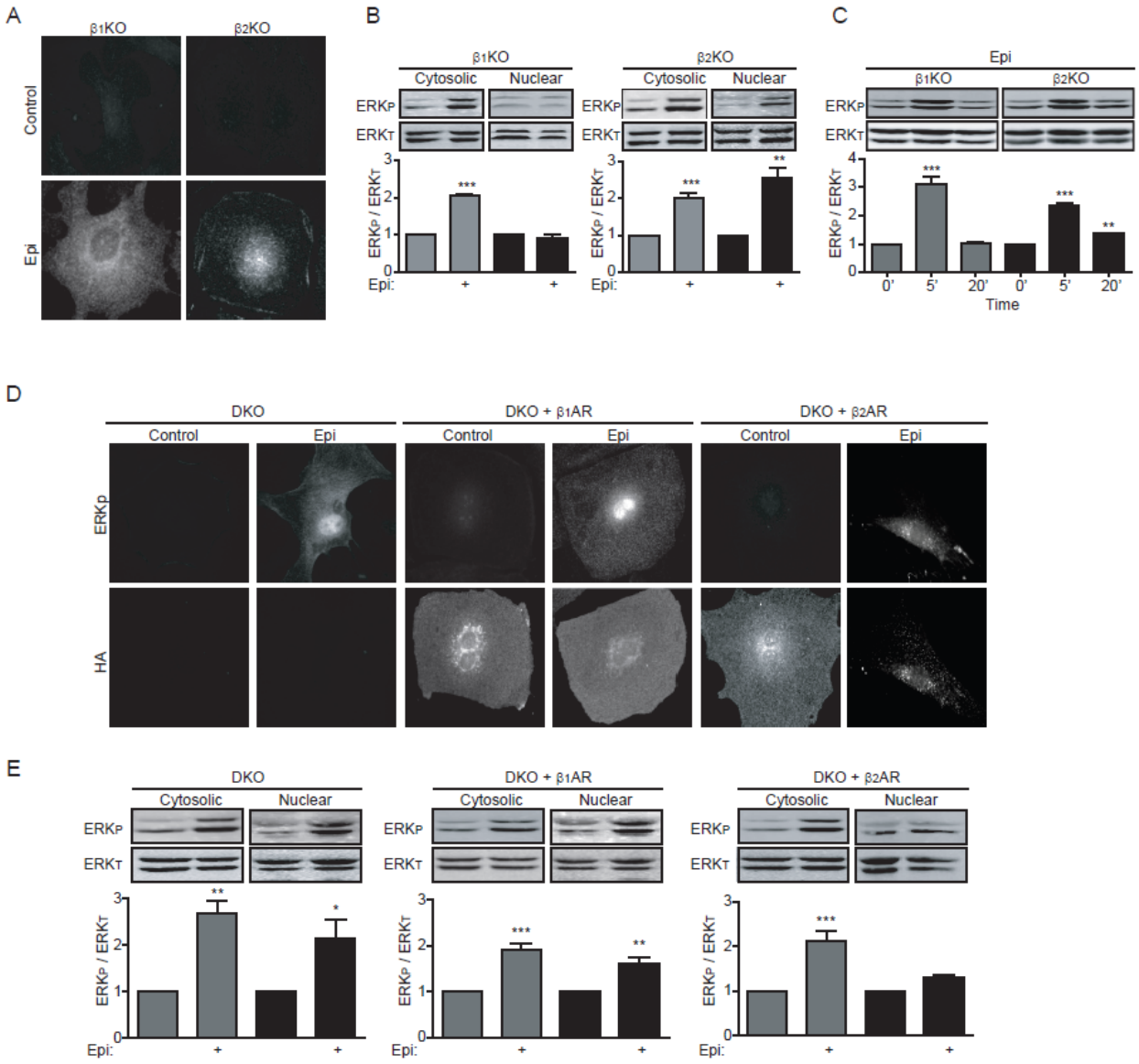


Figure 2.4. The β_2AR, but not the β_1AR, prevents nuclear accumulation of ERK signal induced by α_1ARs. A, Fibroblasts were isolated from mice lacking either the β_1AR (β_1KO) or the β_2AR (β_2KO), cells were stimulated with Epi for 5 min and fixed for phospho-ERK (ERKp) staining. B, β_1KO or β_2KO cardiac fibroblasts were stimulated with Epi for 5 min, the cells were then lysed and separated into nuclear and cytosolic fractions. C, β_1KO or β_2KO cardiac fibroblasts were stimulated with the indicated times. D, Fibroblasts isolated from mice lacking both the β_1AR and β_2AR genes (DKO) were transfected with either HA- β_1AR or HA- β_2AR. Cells were stimulated with Epi for 5 min and fixed for phospho-ERK (ERKp) staining. E, DKO fibroblasts expressing either HA-β_1AR or HA-β_2AR were stimulated with Epi for 5 min, the cells were then lysed and separated into nuclear and cytosolic fractions. Phospho-ERK (ERKp) was normalized against total ERK_T. N=3; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ by unpaired student *t*-test.

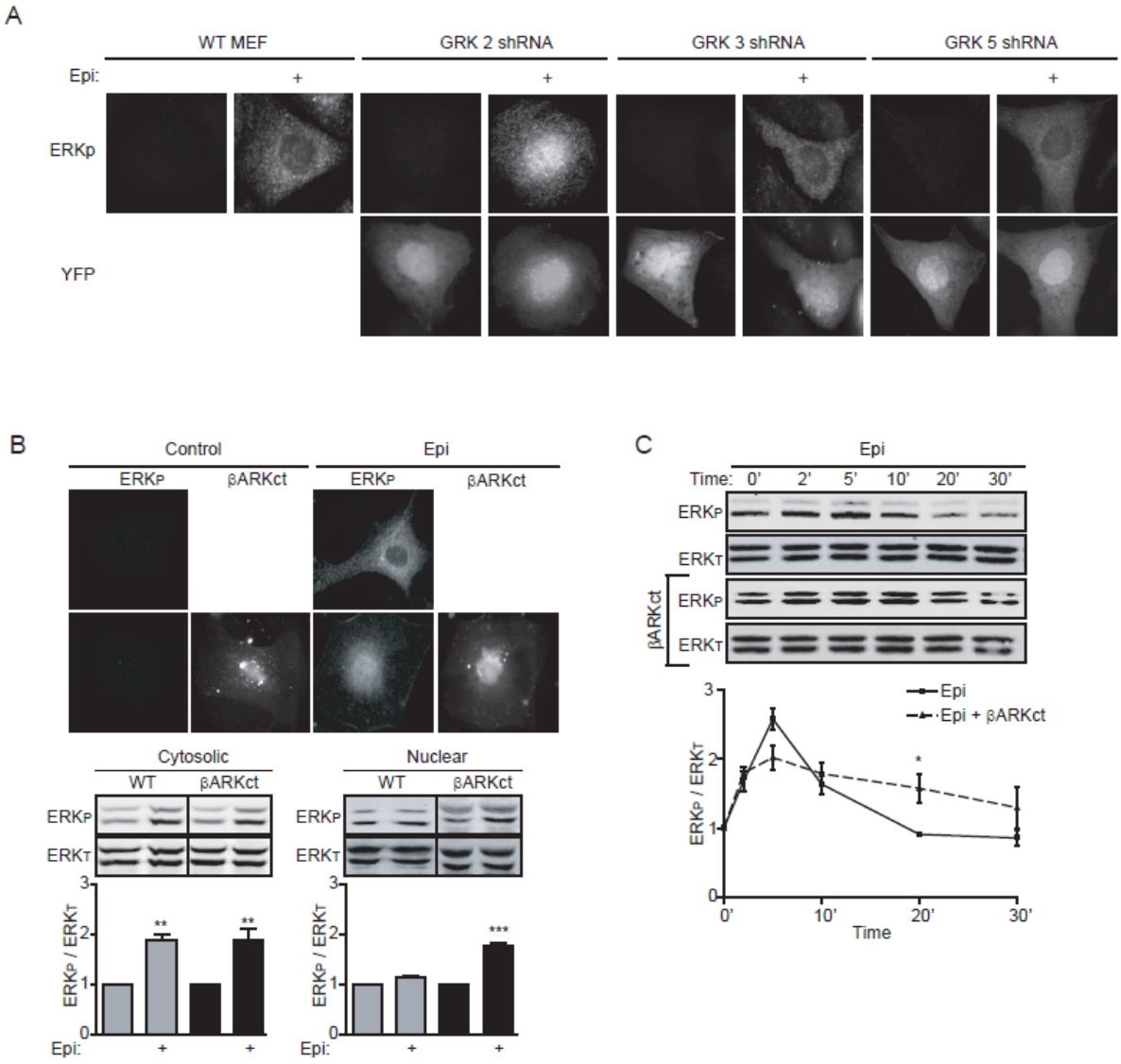


Figure 2.5. GRK2, but not GRK3, mediates β_2AR activation-dependent sequestration and attenuation of α_1AR-induced ERK signal. A, WT cardiac fibroblasts expressing GRK2, GRK3 or GRK5 shRNA were stimulated with Epi (10 μM) for 5 min and fixed for phospho-ERK (ERKp) staining. B, WT cardiac fibroblasts expressing HA-tagged βARKct were stimulated with Epi (10 μM) for 5 min, the cells were either fixed for phospho-ERK (ERKp) staining or lysed and separated into nuclear and cytosolic fractions. C, WT cardiac fibroblasts expressing HA-tagged βARKct were stimulated with Epi (10 μM) the indicated times. Phospho-ERK (ERKp) was normalized against total ERK_T. N=3. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ by unpaired student *t*-test.

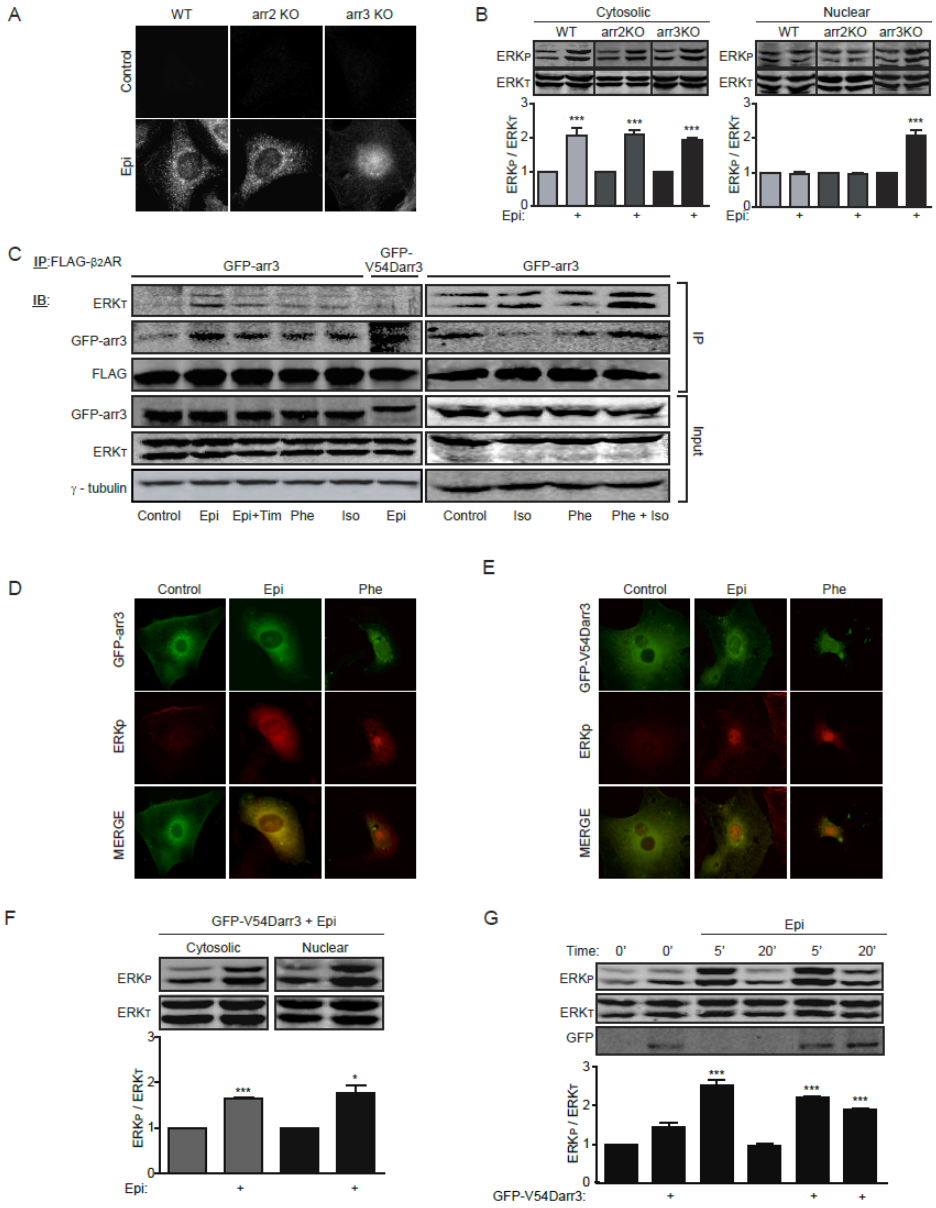


Figure 2.6. β_2AR activation-dependent recruitment of arrestin 3 mediates sequestration and attenuation of α_1AR-induced ERK signal. A, WT, arr2 KO, and arr3 KO MEFs were stimulated with Epi (10 μM) for 5 min and fixed for phospho-ERK (ERKp) staining. B, Epi (10 μM, 5 min) stimulated cells were lysed and separated into nuclear and cytosolic fractions. C, WT fibroblasts expressing flag-β_2AR together with either GFP-Arr3 or dominant negative GFP-V54DArr3 were stimulated with Iso (10 μM), Epi (10 μM), or Phe (10 μM) for 10 minutes after pretreatment with Tim (10 μM) for 5 minutes, if indicated. Cells were lysed for immunoprecipitation with an anti-flag antibody to examine the association between β_2AR/arrestin complex and ERK. D and E, WT fibroblasts expressing GFP-Arr3 or GFP-V54DArr2 were stimulated with Epi for 5 min before being fixed for phospho-ERK (ERKp) staining. F, WT fibroblasts expressing GFP-V54DArr3 were stimulated with Epi (10 μM) for 5 min, the cells were then lysed and separated into nuclear and cytosolic fractions. G, WT fibroblasts expressing GFP-V54DArr3 were stimulated with Epi (10 μM) for the indicated times. Phospho-ERK (ERKp) was normalized against total ERK_T. N=3. *, $p<0.05$; ***, $p<0.001$ by unpaired student *t*-test.

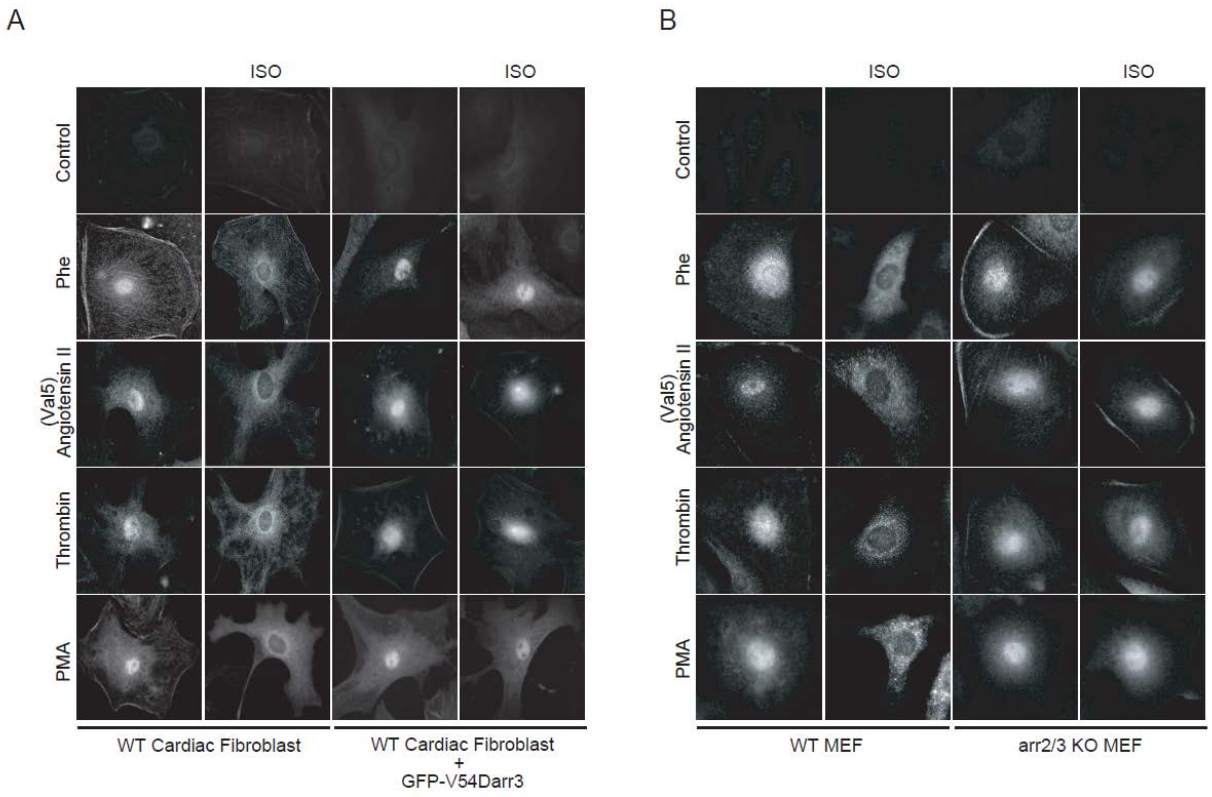


Figure 2.7. Recruitment of β Arrestin upon activation of the β2AR sequesters and Gq-coupled receptor signaling-induced ERK signal within the cytoplasm in cardiac fibroblasts. WT (A) cardiac fibroblasts or (B) MEFs were stimulated with Phe (10 μM), angiotensin II (1 μM), thrombin (1 nM), or PMA (10 μM), in the presence or absence of Iso (10 μM), for 5 minutes. Cells were fixed and stained with anti-ERKp antibody. Alternatively, (A) cardiac fibroblasts expressing a dominant negative GFP-V54DArr2 or (B) MEFs lacking both Arrestin 2 and Arrestin 3 (Arr2/3 KO) were stimulated with Phe (10 μM), angiotensin II (1 μM), thrombin (1 nM), or PMA (10 μM), in the presence or absence of Iso (10 μM), for 5 minutes. Cells were fixed and stained with anti-ERKp antibody.

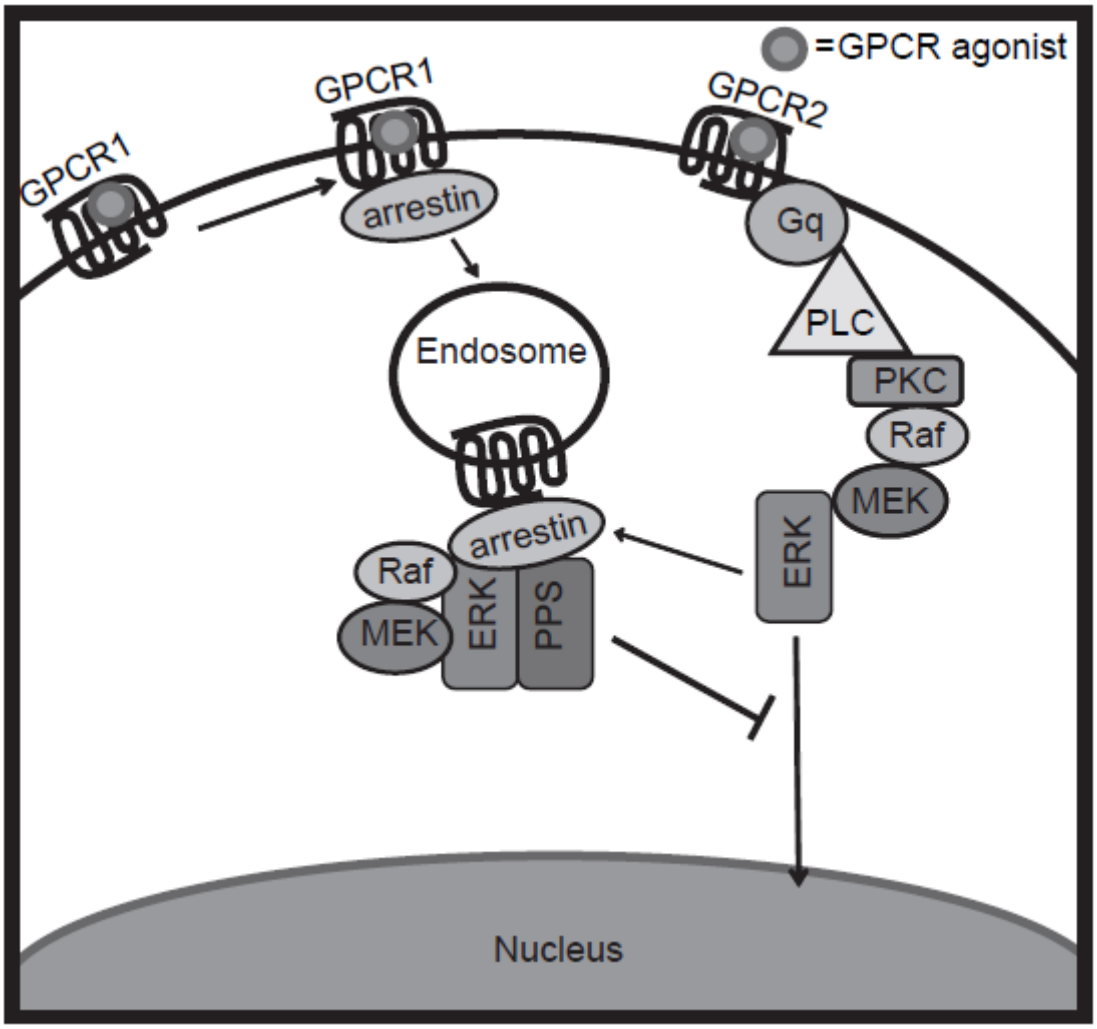
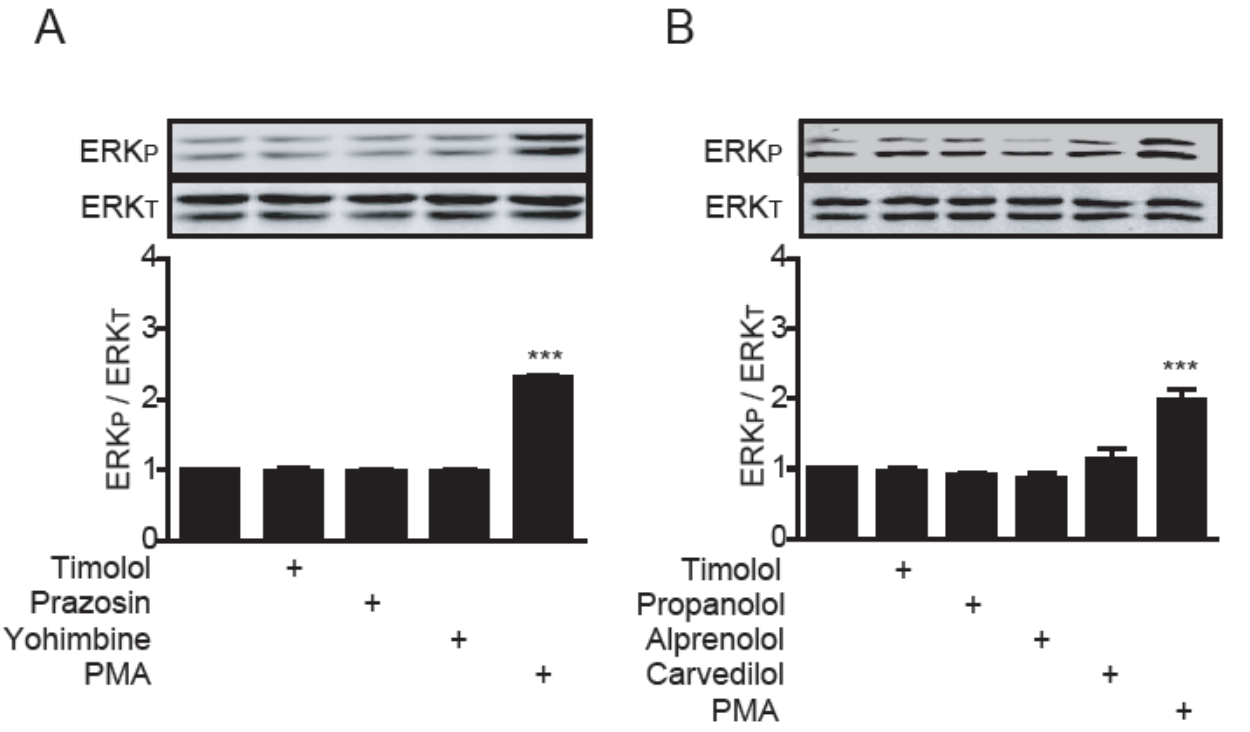
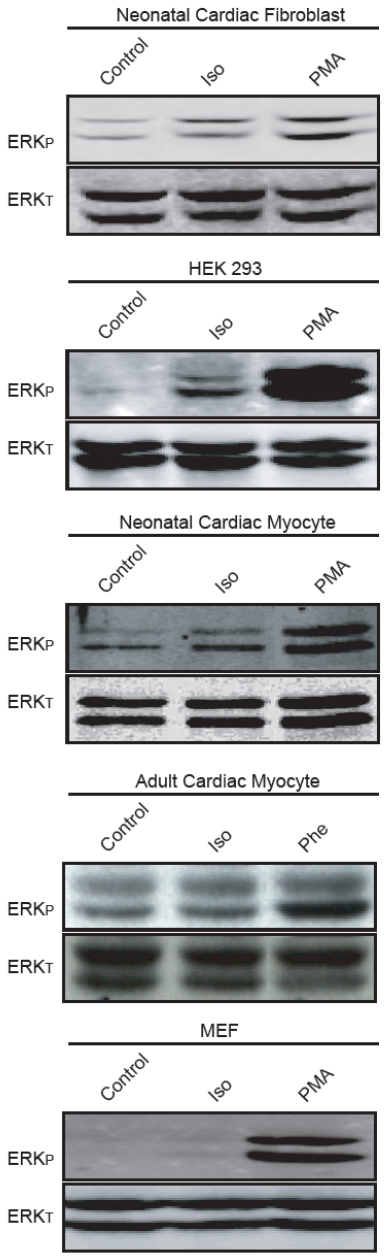


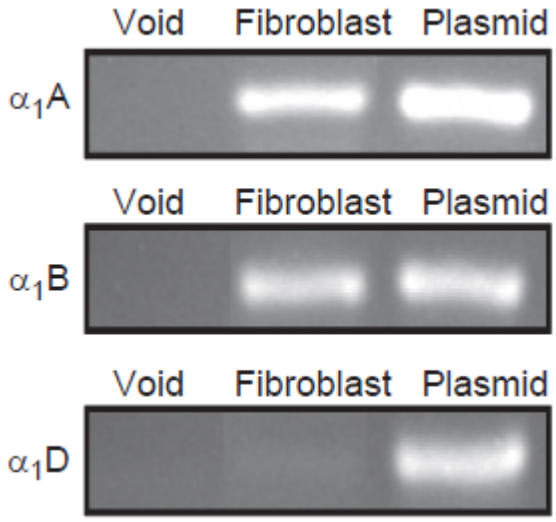
Figure 2.8. Model explaining the mechanism behind GPCR-mediated arrestin-dependent sequestration of ERK induced by another GPCR. Activation of GPCR1 (β_2AR) leads to arrestin recruitment and subsequent β_2AR internalization. The β_2AR-bound arrestin assembles a signaling complex, which recruits Gq-coupled receptor (GPCR2)-induced ERK. Arrestin can potentially "prime" phosphatases (PPS) to dephosphorylate α_1AR-induced ERK. This arrestin-ERK complex sequesters α_1AR-induced ERK within the cytoplasm and prevents nuclear translocation.



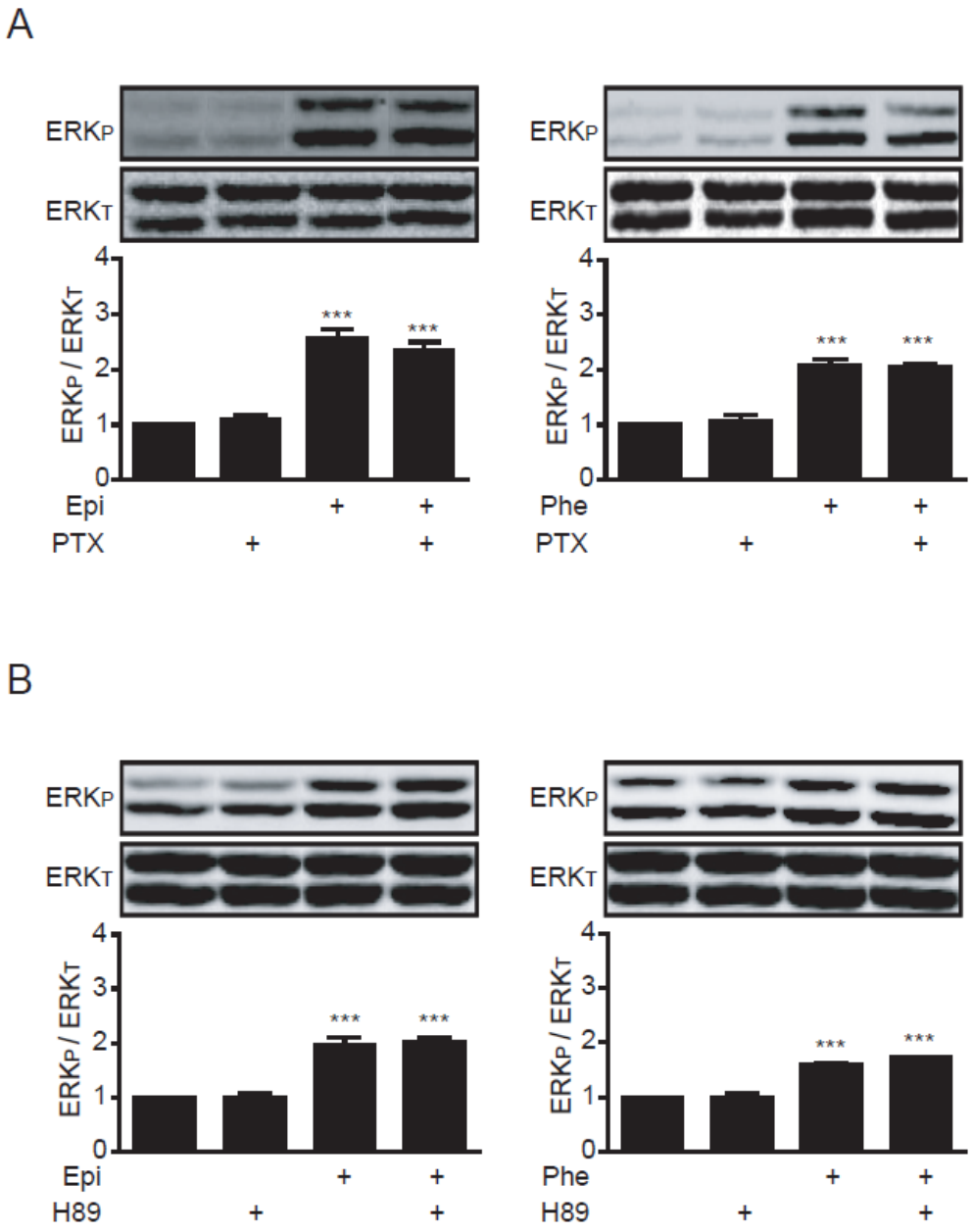
Supplementary Figure 2.I. Adrenergic receptor antagonists do not alter basal phospho-ERK levels. A, WT cardiac fibroblasts were treated for 5 min with 10 μM of either prazosin (Prz) or timolol (Tim) or yohimbine (Yoh). B, WT cardiac fibroblasts were treated for 5 min with 10 μM of either Tim, Propanolol (Pro), Alprenolol (Alp), Carvedilol (Carv) or PMA for 10 min. Cells were harvested and Phospho-ERK (ERKp) and total ERK (ERK_T) were detected with western blotting and signals were normalized as $ERKp/ERK_T$. N=3.



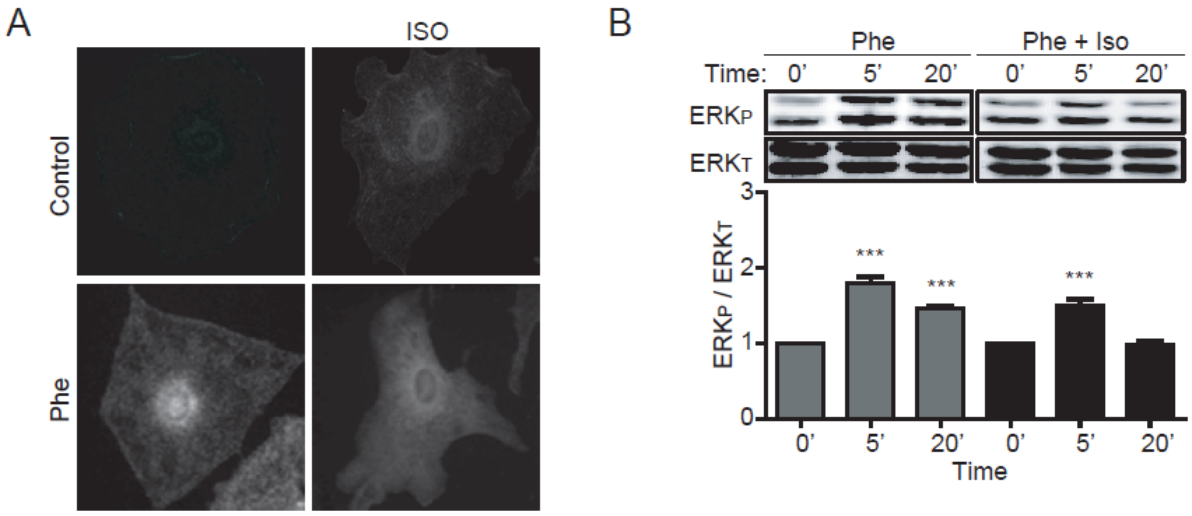
Supplementary Figure 2.II. Iso-induced ERK signaling is both cell- and development specific. Neonatal cardiac fibroblasts, HEK 293 cells, Neonatal cardiac myocytes, Adult cardiac myocytes and MEF cells were treated for 5 min with 10 μM of Isoproterenol (Iso) or 10 min with 10 μM PMA for 10 min. Cells were harvested and Phospho-ERK (ERKp) and total ERK (ERK_T) were detected with western blotting.



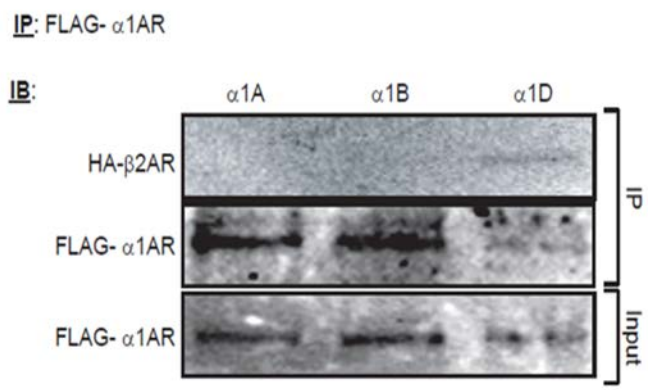
Supplementary Figure 2.III. Neonatal Cardiac fibroblasts predominantly express α_1A and α_1B but not α_1D α1AR subtypes. RNA was extracted from neonatal cardiac fibroblasts according to protocol supplied with the Aurum total RNA minikit (Biorad, Hercules, CA). cDNA was created and amplified via PCR in a one step method using the protocol supplied with the Access RT-PCR system (Promega, Madison, WI). Both α_1A-AR and α_1B-AR, but not α_1D-AR, were detected in neonatal cardiac fibroblasts.



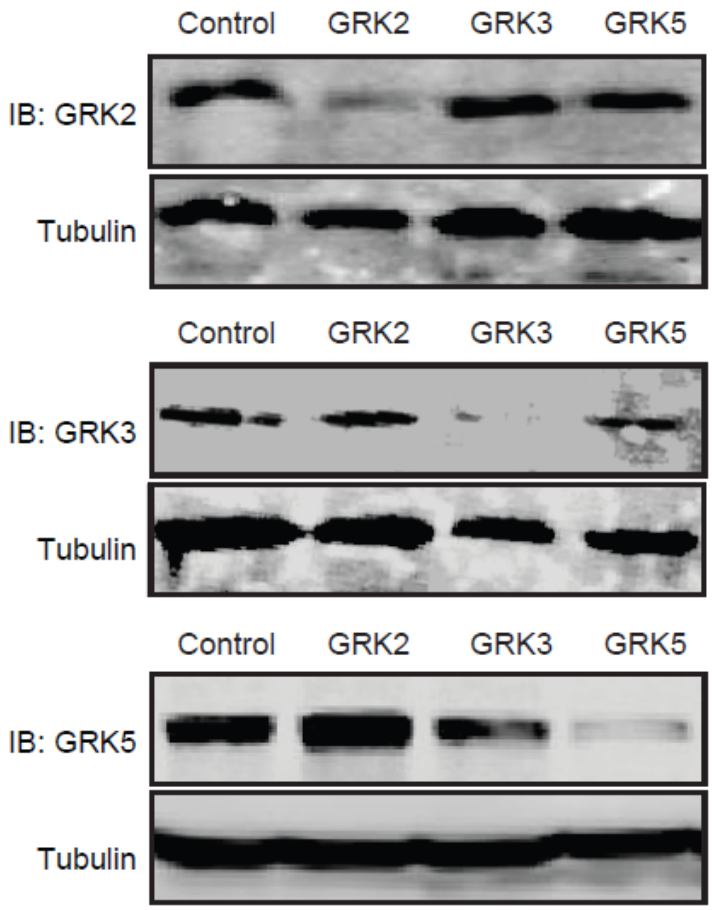
Supplementary Figure 2.IV. ERK activation does not occur via classical Gs-PKA signaling. A, WT cardiac fibroblasts were pretreated, if indicated, with 200 ng of pertussis (PTX) O/N before stimulation with either 10 μM epinephrine or phenylephrine (Phe) for 5 min. B, WT cardiac fibroblasts were pretreated, if indicated, with 10 μM H89 for 30 min before stimulation with either 10 μM epinephrine or phenylephrine (Phe) for 5 min. Cells were harvested and Phospho-ERK (ERKp) and total ERK (ERK_T) were detected with western blotting and signals were normalized as $ERKp/ERK_T$. N=3.



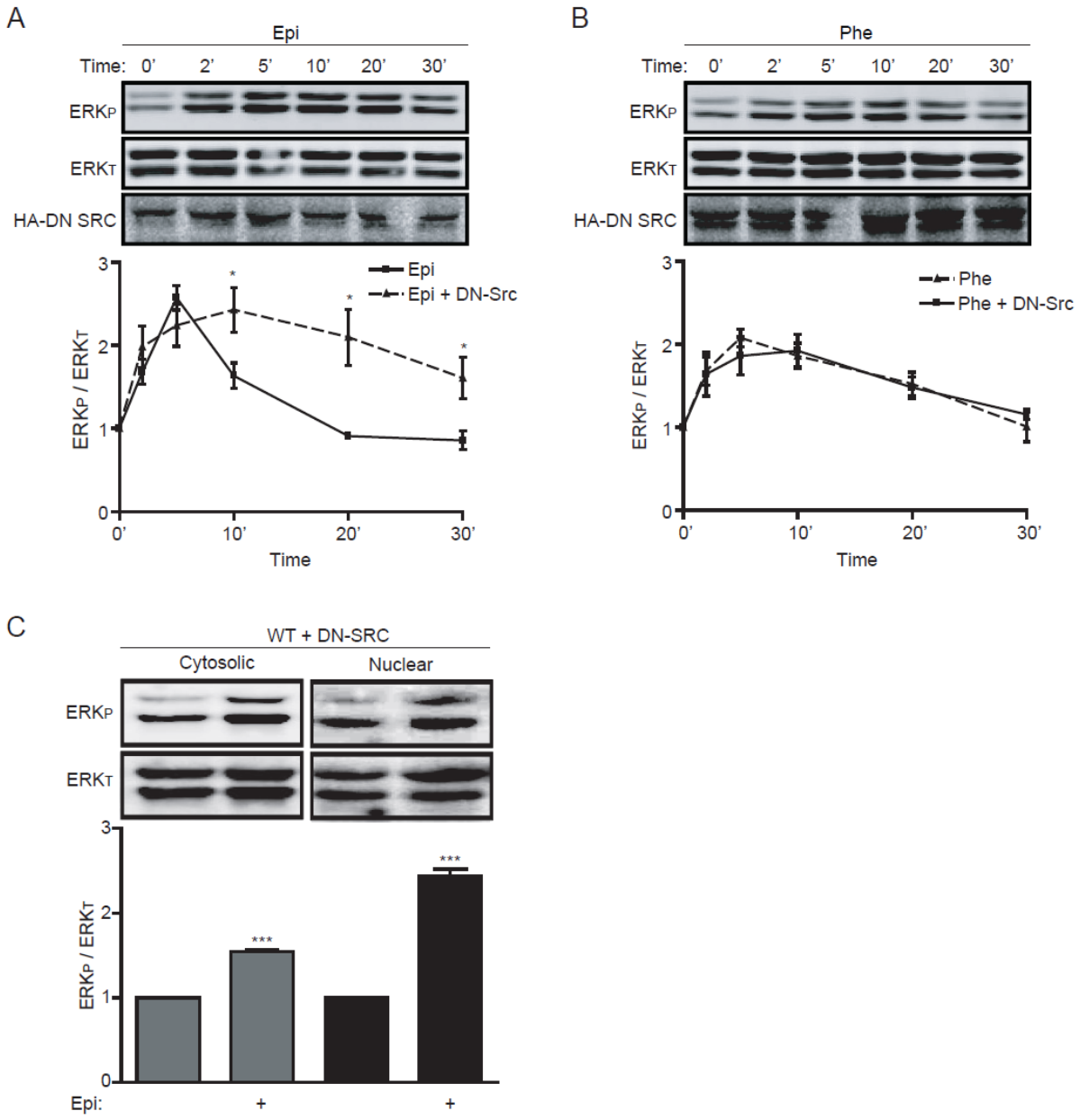
Supplementary Figure 2.V. Co-activation of βARs prevents nuclear accumulation of ERK signal induced by α_1ARs. A, WT cardiac fibroblasts were stimulated with Phe (10 μM) in the presence or absence of Iso (10 μM). Phospho-ERK (ERKp) was detected by immunofluorescence imaging. B, WT fibroblasts were stimulated with Phe (10 μM) in the presence or absence of Iso (10 μM) for 5 mins. Phospho-ERK (ERKp) and total ERK (ERK_T) were detected with Western blotting and signals were normalized as $ERKp/ERK_T$ N=3; ***, $p<0.001$.



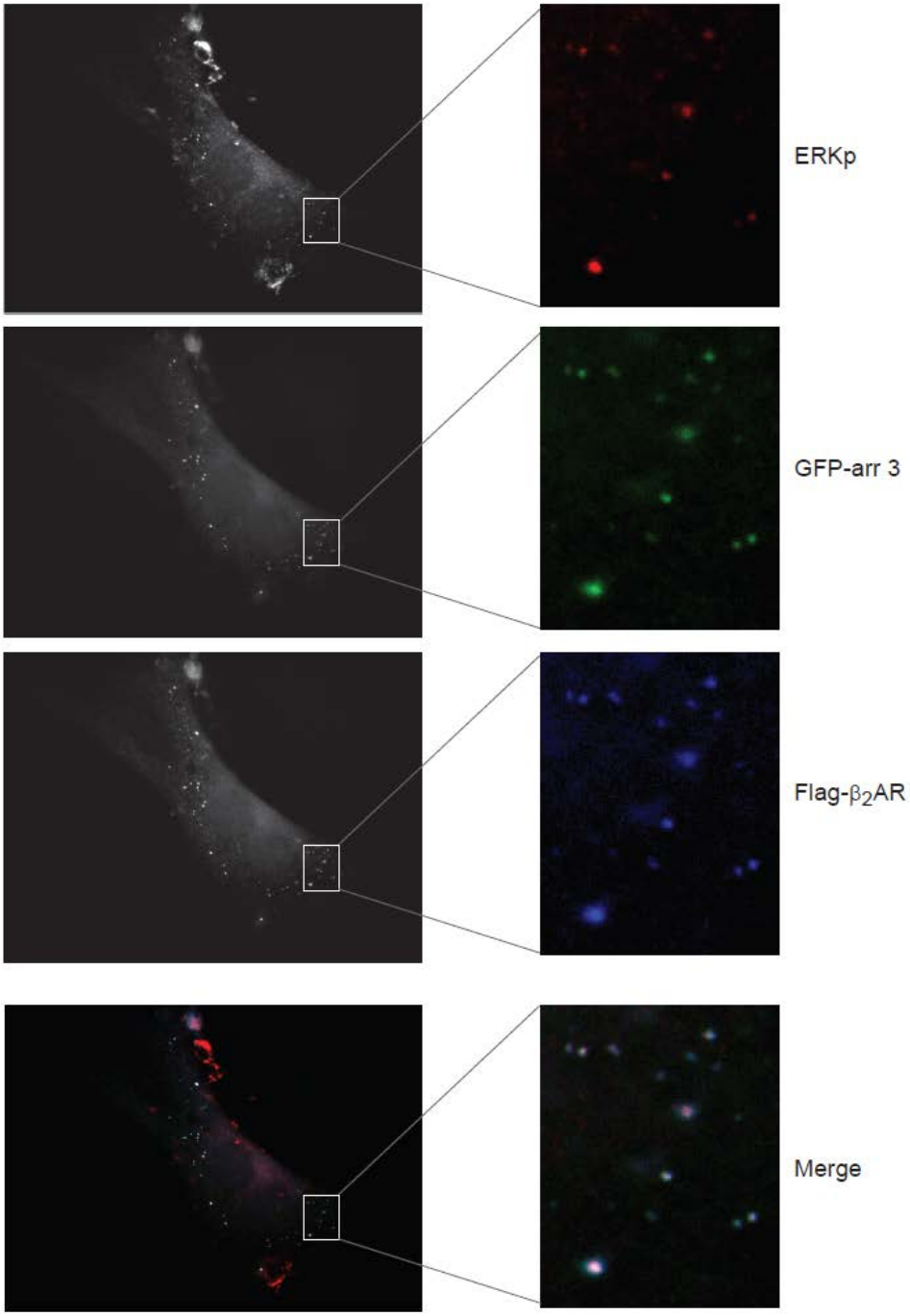
Supplementary Figure 2.VI. α_1ARs do not form heterodimers with β_2AR. FLAG-tagged α_1A-AR, α_1B-AR or α_1D-AR constructs were transfected into MEF cells along with HA-β_2AR. After 48 hr expression, cells were lysed for immunoprecipitation with an anti-flag antibody to examine the association between β_2AR and α1AR subtypes and ERK. HA and FLAG were detected with western blotting.



Supplementary Figure 2.VII. Effective GRK knockdown in MEF cells. WT MEF cells were transfected with shRNA constructs target at either GRK2, GRK3 or GRK5. After expression and selection, cells were lysed and harvested and lysates were subjected to SDS-PAGE. Individual GRK isoforms were visualized with Western blotting.



Supplementary Figure 2.VIII. Src is involved in βAR cross-talk with the α1AR in cardiac fibroblasts. A and B, WT fibroblasts expressing a dominant negative Src (HA-DN-Src) were stimulated with 10 μM Epi or Phe for the indicated times. Phospho-ERK (ERKp) and total ERK (ERK_T) were detected via Western blotting and signal was quantitated as $ERKp/ERK_T$. C, WT fibroblasts expressing a dominant negative Src (HA-DN-Src) were stimulated with Epi for the indicated times. Cells were lysed and separated into nuclear and cytosolic fractions. Phospho-ERK (ERKp) and total ERK (ERK_T) in each fraction were detected via Western blotting and signal was quantitated as $ERKp/ERK_T$ N= 4. *, $p<0.05$; ***, $p<0.001$.



Supplementary Figure 2.IX. β_2AR, Arrestin 3 and ERKp colocalize intracellularly upon Epi stimulation. DKO MEF cells were cotransfected with FLAG-β_2AR and GFP-Arr3 constructs. Cells were then stimulated for 10 min with Epi (10 μM), then fixed and stained with either a FLAG or anti-ERKp antibody.

Chapter 3:

Receptor tyrosine kinases partner with GPCRs to tune mitogen dose-dependent cell proliferation

Abstract

Receptor tyrosine kinases (RTKs) and G-protein coupled receptors[(1)] are two major families of cell surface receptors that are capable of eliciting mitogenic signaling in response to extracellular stimuli. Here, we characterize a novel signaling complex of mitogen-activated insulin receptor and the sympathetic activity-regulated β_2 adrenergic receptor (β_2AR) that fine-tunes insulin dose-dependent subcellular activation of mitogen-activated protein kinase (MAPK) for cell proliferation. Low concentrations of insulin lead to the classic shc/grb2 pathway for activation and nuclear translocation of ERK, and cellular proliferation. Interestingly, increasing concentrations of insulin switch to a β_2AR-dependent pathway via an IR-IRS2-G-protein receptor kinase 2 (GRK2) cascade for phosphorylation and transactivation of the β_2AR. The phosphorylated β_2AR then activates β arrestin 2 for the GPCR internalization and activation of MAPK in the cytoplasm, which attenuates IR-mediated cellular proliferation. Moreover, insulin-like growth factor receptor, but not other RTKs share the same signaling mechanism with IR. Co-stimulation of β_2AR and IR sequesters ERK signal in the cytoplasm induced by low concentrations of mitogen, and prevents IR-induced cell proliferation and IGF-1-induced embryonic stem cell self-renewal. This elaborate, dynamic, and mitogen dose-dependent activation of the IR/β_2AR complex represents a new paradigm in RTK signaling and provides a mechanism for modulation of RTK-dependent cell proliferation by the sympathetic nervous system.

Introduction

Understanding how cells integrate information from a variety of chemically diverse signals into complex orchestrated responses such as cell proliferation, differentiation, and apoptosis is an overarching goal of cell biology. Receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) represent two major families of cell surface receptors to transduce extracellular stimuliand induce heterologous signal transduction pathways leading to divergent and even opposing cellular processes in cells. Although insulin receptor (IR) and β_2 adrenergic receptor (β_2AR) elicit distinct cellular signaling pathways via independent mechanisms, they are involved in overlapping physiological functions by targeting to the same cells in tissues like fat, liver, skeletal muscles, and cardiac muscles. Extensive studies show that insulin and adrenergic activation not only share many components in the intracellular signaling pathways[2-8], but also act reciprocally to blunt each other's signaling in adipocytes and liver cells[9-16]. However, the mechanism underlying the coordination and integration of IR and β_2AR signaling remains poorly understood.

IR, a member of the large RTK family, induces activation of both mitogen-activated protein kinases (MAPKs) and Akt. Activation of Akt is dependent on recruitment and phosphorylation of insulin receptor substrate[17] and activation of PDK and PI3K. Akt regulates glucose uptake, cell survival, and protein synthesis[18-20]. In parallel, IR activation induces Shc/grb2-mediated ERK activity leading to cellular hypertrophy, proliferation, and survival[21-24]. Conversely, ligand binding to β_2AR, a member of the GPCR superfamily, leads to G protein activation, which induces production of second messengers like cAMP for activation of downstream kinase protein kinase A (PKA). The activation of PKA leads to a wide range of stress responses including muscle contraction and relaxation[25], neuron excitation[26-28], and

secretion[29]. In addition to G protein signaling, β_2AR stimulation is also implicated in mitogenic signaling via an arrestin-dependent mechanism[30, 31].

Extensive studies show that insulin and adrenergic activation also act reciprocally to blunt each other's signaling in adipocytes and liver cells. In adipocytes, IR activation leads to increased βAR down-regulation via phosphorylation of the β_2AR[9-14]. Meanwhile, insulin-like growth factor 1 (IGF-1) induces PI3K/AKT-dependent phosphorylation of β_1AR for internalization in CHO cells[32]. On the contrary, chronic stimulation of βAR impairs insulin signaling-induced Akt activation and translocation of glucose transporter 4 (Glut4), as well as glucose uptake in adipocytes and cardiac myocytes[16, 33].

Meanwhile, numerous studies indicate potential coordination of signaling processes between IR and βAR. Recent studies suggest βARs transactivate a growing list of RTKs including the epidermal growth factor receptor[34-36], platelet-derived growth factor receptor[37, 38], and insulin-like growth factor receptor[11, 39]. Alternatively, proteins in βAR signaling pathways also participate in RTK signaling cascades. For example, knockdown of $G\alpha i_2$ in adipose and liver tissue with antisense oligos impairs insulin function, induces hyperinsulinemia, impaired glucose tolerance, and insulin resistance *in vivo*[2]. Furthermore, IGF-1-mediated MAPK phosphorylation is dependent on Gαi/βγ signaling[3, 40] or β arrestin 1 activation[41-43]. Moreover, GRKs[4, 5] and the scaffold/adaptor protein β arrestin[6-8] regulate insulin-induced and GLUT4 dependent glucose uptake.

Due to the high number of parallels between IR and βAR signaling involving common intermediate signaling proteins as well as reciprocal antagonized effects on individual pathways, we envision the potential for RTK-GPCR cross talk via integrated signaling complexes to coordinate efficiency and specificity of signaling for divergent cellular responses. Here we have

characterized novel signaling machinery in which IR and β_2AR form a complex to fine-tune subcellular activation of IR-induced mitogenic signaling. Stimulation of IR leads to IRS2-mediated GRK recruitment that is capable of phosphorylating the β_2AR leading to β arrestin 2-mediated β_2AR internalization. The activation of β_2AR/β arrestin 2 is necessary to promote full activation of mitogenic signaling, which is also sequestered in the cytoplasm. In contrast the classic mitogenic signaling induced by IR via Shc/Grb2 activation is small, and enriched in the nucleus. IR activates these two distinct pathways in an insulin dose-dependent fashion, through which low concentration promotes the classic pathway and cell proliferation, whereas high concentration leads to transactivation of β_2AR-dependent mitogenic activity. Moreover, we show that only IR and IGF-R utilize β_2AR to induce mitogenic signaling whereas other RTKs do not. Co-stimulation of β_2AR convertsthe classic nuclear mitogenic signaling into a β_2AR-dependent cytoplasmic signaling, and blocks insulin-induced cell proliferation and Insulin-like Growth Factor (IGF-1)-induced mouse embryonic stem cell self-renewal. The discovery that RTKs utilize GPCR machinery to induce subcellular activation of mitogenic signaling in a mitogen dose-dependent fashion is a novel paradigm in RTK signal transduction, presenting a new facet of RTK signaling. Our results also underscore the critical role of signaling cross talk and integration between RTKs and GPCRs *in vivo*.

Materials & Methods

Cell culture, transfection, and adenoviral infections

Animal protocols were approved by the IACUC of the University of Illinois according to NIH regulation. H9C2 rat myoblast cell lines were obtained from ATCC. Mouse Embryonic Fibroblasts (MEFs) were isolated from the embryos of wild-type, β_1AR knock-out (KO), β_2AR

KO or β_1/β_2AR DKO FVB mice. MEFs from β-arrestin 1 KO, β arrestin 2 KO, and β arrestin 1/β arrestin 2 DKO mice were donated by Dr. Robert Lefkowitz (Duke University). MEFs from Gi1 KO, Gi2 KO, Gi3 KO mice were described else where[44]. MEFs from IRS1 KO, IRS2 KO or IRS1/2 DKO mice were gifts from Dr. Morris White (Harvard University).

Cells were cultured in T75 tissue culture treated flasks until 90% confluent. Cells were then plated on 35 mm or 10 cm tissue culture treated dishes at a confluency of 60-70%. MEFs were maintained for 24h in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and experiments were performed, unless otherwise noted, on cells serum-deprived for 24h. H9C2 cardiac myoblasts were cultured in T75 tissue culture treated flasks until 90% confluent. Cells were then plated on 35 mm or 10 cm tissue culture treated dishes at a confluency of 60-70%. H9C2 myoblasts were maintained for 24h in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and experiments were performed, unless otherwise noted, on cells serum-deprived for at least 3h.

MEFs or H9C2 cardiac myoblasts were infected with recombinant adenoviruses or transfected, as indicated, after being cultured for 24h. Recombinant plasmids expressing βARKct, GFP- β arrestin 2, and GFP- β arrestin 2 V54D were generated as previously described[1]. GFP- β-arrestin 2 and GFP- β arrestin 2 V54D plasmids were gifts from Dr. Gang Pei (Chinese Academy of Sciences, Shanghai). Plasmids containing HA-tagged GRK2 mutants lacking the Pleckstrin homology domain (ΔPH) or the kinase domain (ΔKinase) were constructed in pcDNA3 (Invitrogen, CA). Experiments were conducted after 48h expression.

Drug Treatment

Cells were treated with the following agonists or antagonists at the indicated dosage and times: the IR agonist insulin (Sigma, St. Louis, MO), the βAR agonist isoproterenol (Sigma, St.

Louis, MO), the α_1AR agonist phenylephrine (Sigma, St. Louis, MO), the PKC stimulator phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO), insulin-like growth factor 1 (IGF; Sigma, St. Louis, MO), epidermal growth factor (EGF; Sigma, St. Louis, MO), platelet-derived growth factor (PDGF; Peprotech, Rocky Hill, NJ), fibroblast growth factor (FGF; Sigma, St. Louis, MO) or nerve growth factor (NGF; Sigma, St. Louis, MO). Cells were treated with the following inhibitors as indicated: an AKT inhibitor LY294002 (Sigma, St. Louis, MO), a Shc/Grb2 inhibitor actinomycin D (Sigma, St. Louis, MO), a Gi specific inhibitor pertussis toxin (PTX; Sigma, St. Louis, MO), a non-specific phospholipase C (PLC) inhibitor U73122 (Calbiochem, San Diego, CA), a PKA inhibitor N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89; Sigma, St. Louis, MO), a PKC inhibitor calphostin C (Calbiochem, San Diego, CA), or a MEK inhibitor U1026 (Sigma, St. Louis, MO).

Immunoblotting

Unless specifically noted, all antibodies were purchased from Santa Cruz (Santa Cruz, CA). Antibodies specific to both phosphorylated and total ERK1/2 were purchased from Cell Signaling (Danvers, MA). Cells were serum-starved for 24h prior to the addition of the agonist or antagonist as indicated. Alternatively, cells were pretreated with inhibitors as indicated in the figure legends. After stimulation, cells were chilled, washed, and harvested in lysis buffer (5 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% NP-40, and Halt Protease/Phosphatase Inhibitor Cocktail from (Thermo Scientific, IL). Lysates were clarified and resolved on 10% SDS-PAGE gels for Western blot. Phosphorylated and total ERK were detected via incubation with the corresponding antibodies at 1:1000 for 2h RT. Other antibodies were used at 1:800 for 2h at RT unless otherwise noted. Primary antibodies were visualized with IRDye 680CW goat anti-mouse or with IRDye 800CW goat anti-rabbit secondary antibodies using an Odyssey scanner

(1:10,000; LI-COR Biosciences, Lincoln, NE). Phospho-ERK signals were corrected for total ERK levels and plotted as increase over basal levels. Alternatively, S355/S356 phosphorylation levels were corrected against γ-tubulin levels and plotted as arbitrary units (AU). Signal intensity was quantitated by densitometry of Western blots.

Immunofluorescence microscopy

Fluorescent images were taken with a Zeiss Axioplan 2 microscope (Carl Zeiss Inc., Thornwood, NY) with an attached CCD camera and Metamorph software (Molecular Devices, Sunnyvale, CA). Cells were plated on laminin-coated coverslips and then serum starved for 24h. Cells were then stimulated as indicated and then fixed with 4% paraformaldehyde for 1h at 4^{o}C. Cells were then permeabilized with PBST (0.1% Triton X-100 in PBS) for 15 min at RT, rinsed and blocked, and then incubated with 1:400 IR antibody, 1:400 β_2AR antibody, or 1:400 ERKP antibody diluted in blocking buffer (PBST with 10% FBS) for 2h at RT. AlexaFluor 594-conjugated goat anti-mouse IgG1 antibody, AlexaFluor 488-conjugated goat anti-rabbit IgG1, or AlexaFluor 350-conjugated goat anti-rabbit IgG1 antibodies were utilized as the secondary antibody diluted to 1:1000 in blocking buffer for 1h at RT. Alternatively, cells were infected with recombinant plasmids expressing GFP- β-arrestin 2 to allow visualization.

Co-immunoprecipitation

Wild-type H9C2, WT MEF, and IRS2 KO MEF cells were utilized for co-immunoprecipitation. WT H9C2 cells were infected with both FLAG-tagged mouse β_2AR and GFP- β arrestin 2 before the procedure. Cells were stimulated with the indicated agonist for 10 min before being lysed with lysis buffer. Lysates were cleared by centrifugation and subjected to immunoprecipitation with either M2 anti-FLAG affinity resin (Sigma, St. Louis, MO) or Protein A beads (Pierce, Rockford, IL) coated with IR IRS2 or GRK2 antibodies. The

immunoprecipitates were resolved via SDS-PAGE and blotted with antibodies against IR (1:500), β_2AR (1:500), β arrestin 2 (1:500), IRS2 (1:500), GRK2 (1:1000), GRK3 (1:1000), HA (1:500), GFP (1:1000) or Gi (1:500). Primary antibodies were visualized with IRDye 680CW goat anti-mouse or with IRDye 800CW goat anti-rabbit secondary antibodies using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE).

Cell Proliferation and sizing assays

WT, β_1AR KO or β_2AR KO MEF or H9C2 rat myoblast cells were stimulated with the indicated agonist or antagonist for 48h; cells were then lysed and harvested. Total protein concentration was measured via BCA assay. Colorimetric analysis was performed with a Spectramax M2 fluorometer reader (Molecular Devices, Sunnyvale, CA). The data were normalized against the control. Alternatively, protein levels were quantitated by Western blotting against γ-tubulin (1:2000). For cell counting assays, H9C2 cardiac myoblasts were stimulated with the indicated agonist or antagonist for 48h. Pictures were taken with a Zeiss Axioplan 2 microscope (Carl Zeiss Inc., Thornwood, NY) with an attached CCD camera and Metamorph software (Molecular Devices, Sunnyvale, CA). Cells were then counted in a blinded manner. The cell proliferation ELISA was carried out utilizing BrdU Labeling and Detection Kit III (Roche, Basel, Switzerland) according to the manufacturer's instructions. Cells were cultured in a 96-well plate as described above and left overnight to attach. 10 μM BrdU was added to cells prior to drug stimulation for 24h at 37^{o}C. Cells were then fixed and nucleases were allowed to partially digest cellular DNA. Cells were then incubated with an anti-BrdU antibody (200 mU/ml) for 30 min at 37^{o}C. Colorimetric analysis was performed with a Spectramax M2 fluorometer reader. The data were normalized against the control.

GRK and IR Knockdown

GRK2, GRK3, GRK5, and GRK6 mouse shRNA plasmids (Sigma, St. Louis, MO) were used to create recombinant lentiviruses. WT MEF cells were infected with GRK shRNA lentiviruses. After overnight infection and additional 24h expression, 10 μg/ml of puromycin were added to the dishes to select for positive infection. After 48h puromycin selection, cells were serum starved for another 24h. Cells were then harvested as described above for Western blot. Cell lysates were resolved via SDS-PAGE and blotted with antibodies against GRK2, GRK3, GRK5, or GRK6 (1:1000). Primary antibodies were visualized with IRDye 680CW goat anti-mouse or with IRDye 800CW goat anti-rabbit secondary antibodies using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE). Alternatively, siRNA oligos targeting the mouse insulin receptor (Integrated DNA Technologies, Coralville, IA) were transfected into WT MEF cells and experiments were conducted after 48h expression.

Statistical analysis

Data analysis was performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA).

Results

β_2AR modulates IR-induced ERK signaling at high insulin concentrations

Both IRs and βARs are implicated in promoting ERK activation via distinct signaling mechanisms[24, 30, 31]. Utilizing MEFs and H9C2 cardiac myoblast cells, we investigated the mechanism leading to the modulation of ERK signaling upon potential cross talk between the IR and the βARs. We examined agonist-dependent ERK phosphorylation induced by insulin. Insulin stimulation potently activated ERK in a dose-dependent manner (Fig 3.1A) with

phosphorylation levels peaking at 10 min (Fig 3.1B). Further, the localization of phospho ERK was also dose-dependent, switching from the nucleus to the cytoplasm at increasing concentration of insulin (Fig 3.1C). Surprisingly, deletion of the β_2AR, but not the β_1AR significantly reduced insulin-induced ERK signaling (Figs 3.1D and 3.1E) while leaving insulin-induced AKT signaling intact (Supplementary Figure 3.I). Previous studies have shown that β_2AR activation is sufficient to sequester ERK signaling in the cytoplasm[(1)]. We found that stimulation with higher concentrations of insulin (100 nM) resulted in nuclear accumulation of ERK signaling in the absence of the β_2AR (Fig 3.1E). In contrast, at low concentrations of insulin (10 nM), phospho-ERK translocated to the nucleus in the presence or absence of the β_2ARs (Fig 3.1E). Moreover, insulin induced ERK activity is dependent on the IR (Fig 3.1F). Together, these results suggest that β_2AR was necessary for ERK activation and cytoplasmic sequestration in response to IR stimulation. Interestingly, this cross talk occurred in the presence of inhibition of classical β_2AR signaling pathways (Supplementary Figure 3.II). Further, alterations in ERK signaling were not due to altered protein expression of IR and β_2AR signaling components in MEF cells lacking adrenergic receptors (Supplementary Figure 3.III). Thus, while insulin-induced ERK signaling is dependent on β_2AR expression, β_2AR expression alters the spatial ERK signaling profile in an insulin dose-dependent fashion.

Cross talk with the β2AR is a significant pathway contributing to IR-mediated ERK signaling

Classically, ERK signaling originating from the IR occurs though the shc/grb2/SOS/ras/raf/MEK axis[(24)]. We observed the existence of two signaling pathways—one, β_2AR-independent pathway, activated in response to low insulin concentrations and the other, β_2AR-dependent pathway, activated upon high insulin concentrations. We then set to define

these two independent signaling pathways. Inhibition of shc/grb2 with actinomycin D slightly reduced ERK activation upon stimulation with 100 nM insulin (Figs 3.2A and 3.2D), but completely prevented ERK activation upon stimulation with 10 nM insulin (Figs 3.2B and 3.2D). Moreover, actinomycin did not alter the subcellular ERK localization upon stimulation with 100 nM insulin (Fig 3.2D). However, upon deletion of βARs, inhibition of the shc/grb2 interaction with actinomycin D prevented ERK activation with either 100 nM or 10 nM insulin stimulation (Figs 3.2C and 3.2D). Together, these results confirm that, at low insulin concentrations, ERK activation originating from the IR occurs though the classical shc/grb2/SOS/ras/raf/MEK axis leading to nuclear ERK translocation. Alternatively, upon stimulation with 100 nM insulin, a separate and β_2AR-dependent pathway is activated to promote full activation of ERK that is also sequestrated in cytoplasm.

β_2AR-dependent cytoplasmic sequestration of insulin-induced mitogenic signaling occurs via β_2AR/β arrestin 2 scaffold formation.

Activation of β_2AR stimulation induces ERK signal via a β arrestin 2-mediated formation of β_2AR/β arrestin 2/ERK scaffold[30, 31]. We sought to determine whether insulin stimulation induces arrestin-mediated ERK activation though this pathway. Upon 100 nM insulin stimulation, MEFs lacking β arrestin 2 displayed decreased ERK activation overall, which localized within the nucleus (Figs 3.3A and 3.3B). Conversely, cells containing β arrestin 2 displayed robust ERK activation that was localized in the cytoplasm (Figs 3.3A and 3.3B). To confirm the role of β arrestin 2 in insulin-mediated ERK activation, we introduced a dominant-negative β arrestin 2 mutant (β arrestin 2 V54D). Addition of this mutant significantly reduced ERK activation upon 100 nM insulin stimulation (Fig 3.3C). Previous studies indicate that IR can interact with β arrestins in adipocytes[45]. The involvement of the β_2AR as well as β arrestin 2

in insulin-induced ERK activation suggests that the IR and β_2AR may interact on the cell membrane. To confirm this, we examined the association between IR, β_2AR and β arrestin 2 upon 100 nM insulin stimulation. At basal conditions IR associated with β_2AR and β arrestin 2 (Fig 3.3D). Conversely, β_2AR did not interact with β arrestin 2 (Fig 3.3D). Upon stimulation, however, IR lost its interaction with both β_2AR and β arrestin 2 while β_2AR became associated with β arrestin 2 (Fig 3.3D). Furthermore, β_2AR/β arrestin 2/ERK colocalized upon insulin stimulation in a punctate pattern, suggesting arrestin-mediated β_2AR internalization and formation of a β_2AR/β arrestin 2/ERK complex leading to cytoplasmic sequestration of ERK signaling (Fig 3.3E). Together, our data suggest that insulin stimulates β arrestin 2-mediated β2AR internalization and formation of a β2AR/β arrestin 2/ERK complex within the cytoplasm.

GRK2-mediated β_2AR phosphorylation is required for insulin-induced ERK activity

Because IR/β_2AR cross talk occurs despite classical β_2AR signaling component inhibition (Supplementary Figure 3.II), we suspected non-traditional β_2AR signaling. GRK phosphorylation of the β_2AR at serines 355 and 356 is a well-defined mechanism leading to β arrestin 2-mediated receptor internalization and initiation of ERK activation[30]. We hypothesized that IR recruits GRK to phosphorylate the β_2AR leading to β arrestin 2-mediated initiation of the ERK signaling cascade. Selective knockdown of GRK2, but not other GRKs in MEF cells significantly attenuated ERK activation in response to 100 nM insulin stimulation (Fig 3.4A and Supplementary Figure 3.IV). In addition, transfection of WT β_2AR, but not a mutant β_2AR lacking the GRK2 phosphorylation sites, was able to recover insulin-induced ERK signaling in β_2AR KO MEF cells (Fig 3.4B). To examine whether insulin stimulation leads to GRK2-mediated β_2AR phosphorylation, we examined S355/S356 phosphorylation of the β_2AR in response to insulin stimulation. Insulin stimulation led to a time-dependent GRK-mediated

phosphorylation of the β2AR (Fig 3.4C). β_2AR phosphorylation also displayed a biphasic increase in an insulin-dose dependent fahsion with second phase beginning at 100 nM insulin stimulation (Fig 3.4D). These data suggest the presence of two independent signaling pathways existing—the shc/grb2-dependent pathway that is activated upon stimulation with low concentrations of insulin and the β_2AR-dependent pathway that is only activated upon high insulin stimulation. To confirm that phosphorylation of the β_2AR at serines 355 and 356 is dependent on GRK2, we utilized a well-characterized GRK2 inhibitor (βARKct) and examined S355/S356 phosphorylation upon 100 nM insulin stimulation. We found that inhibition of GRK2 with βARKct significantly reduced S355/S356 phosphorylation (Fig 3.4E). Together, these results confirm our hypothesis that β_2AR phosphorylation of S355/S356 results from IR-mediated GRK2 recruitment and that this phosphorylation is necessary for β arrestin 2 recruitment to the β_2AR and for ERK activation.

IRS2 recruits GRK2 to the IR allowing β_2AR phosphorylation

The IR is capable of utilizing both Gi proteins[4] and IRS proteins[5] to recruit GRK proteins. In order to determine the mechanism by which IR recruits GRK2 to phosphorylate the β_2AR, we examined ERK activation in both IRS KO and Gi KO MEF cells. Interestingly, deletion of Gi proteins had no effect on overall ERK activation upon insulin stimulation (Supplementary Figure 3.V). Conversely, deletion of IRS2, but not IRS1, significantly attenuated ERK activation upon 100 nM insulin stimulation (Fig 3.5A). In addition, deletion of IRS2 prevented β2AR phosphorylation at S355/S356 (Fig 3.5B). To further confirm the role of IRS2 in scaffolding GRK2 to the IR, Co-IP experiments showed an increased GRK2/IR/IRS2 tertiary interaction upon insulin stimulation (Figs 3.5C and 3.5D). As predicted, Gi did not associate with this complex. In accordance, deletion of IRS2 prevented GRK2 interaction with

IR both in the presence and absence of insulin stimulation (Fig 3.5E). Together, these data show that IRS2 is important in recruiting GRK2 to the IR.

The C-terminal Pleckstrin Homology domain of GRK2 is essential in recruiting GRK2 to IRS2

Although IRS proteins have been shown to recruit GRK proteins, the exact region of GRK2 necessary for interaction with IRS2 is unknown. In order to investigate this we utilized the following mutant GRK2 proteins: a mutant GRK2 lacking both the N terminal RGS homology[46] domain and the central kinase domain (βARKct), a mutant GRK2 lacking the kinase domain and the C terminal Pleckstrin homology (PH) domain (Δ kinase), or a mutant GRK2 with an N terminal RH domain deletion (Δ RH). Mutant constructs containing the C terminal PH domain significantly attenuated ERK activation upon insulin stimulation, suggesting competitive inhibition of WT GRK2 binding to IRS2 (Fig 3.6A). To confirm the domain of GRK2 necessary for IRS2 interaction, we transfected MEF cells with WT GRK2 or either the mutants (βARKct, Δ RH, or Δ kinase). IRS2 was then immunoprecipitated to examine which mutants were capable of binding IRS2. We found that only mutant GRK2 constructs containing the C terminal PH domain (βARKct and Δ RH) were capable of binding IRS2 (Fig 3.6B). Together, these results confirm that the C terminal PH domain of GRK2 is necessary for recruitment of GRK2 to IRS2 and ERK activation.

β_2AR cross talk is a universal mechanism regulating RTK-mediated ERK activation for cell proliferation

Due to the similarities among different RTK signaling pathways, we examined the potential cross talk with the β_2ARs by different RTK-mediated ERK activation. Surprisingly, β_2AR deletion only attenuated insulin- and IGF-1-mediated ERK activation while stimulation

with other growth factors including EGF, FGF and PDGF was unaffected by β_2AR deletion (Fig 3.7). Therefore, the cross talk with the β_2AR is specific to members of the class II RTKs, including the IR and Insulin-like Growth Factor receptor (IGFR).

Studies have indicated that the localization of ERK within a cell determine the physiological outcome of signaling. We examined cellular proliferation upon IR/β_2AR cross talk. We found that stimulation of H9C2 with 10 nM insulin promoted an increase in protein synthesis (Fig 3.8A), cell proliferation (Fig 3.8B) as well as BrdU incorporation (Fig 3.8C) which was attenuated by ERK inhibition (Figs 3.8A-3.8C). Furthermore, we found that β_2AR deletion enhances MEF protein content (Fig 3.8D) as well as BrdU incorporation (Fig 3.8E) upon stimulation with 100 nM insulin. Interestingly, 10 nM insulin treatment for 48h stimulated significantly more protein production (Fig 3.8F), cell proliferation (Fig 3.8G) and BrdU incorporation (Fig 3.8H) than 100 nM insulin treatment. The increased protein production upon 100 nM insulin stimulation, however, was not due to cell proliferation, suggesting cellular hypertrophy (Fig 3.8I). Together, these results suggest that nuclear translocation of ERK is necessary for cellular proliferation in both H9C2 cells as well as MEF cells.

Stimulation of β_2AR prevents cell proliferation by redistributing cellular ERK induced by mitogen stimulation

Previously we have shown that β_2AR stimulation is capable of sequestering ERK signaling originating from other GPCRs via β_2AR/β arrestin 2/ERK complex formation[(1)]. We found that β2AR stimulation with isoproterenol prevented nuclear translocation of IR-induced ERK upon 10 nM insulin stimulation (Fig 3.9A). Accordingly, isoproterenol pretreatment significantly reduced H9C2 protein synthesis (Fig 3.9B), cell proliferation (Fig 3.9C), and BrdU incorporation (Fig 3.9D). In addition, isoproterenol pretreatment attenuated insulin-induced

protein production, cellular proliferation, as well as BrdU incorporation in WT MEF cells (Figs 3.9E-3.9G). As expected, β_2AR deletion prevented isoproterenol-induced attenuation of protein production, cellular proliferation, and BrdU incorporation under insulin sitmulation (Figs 3.9E-3.9G). In order to determine whether βAR stimulation-induced attenuation of RTK-induced cell proliferation is a universal mechanism, we examined the proliferation of mouse embryonic stem cells (mESCs). Interestingly, we found that βAR stimulation attenuated IGF1-induced mESC protein synthesis (Fig 3.9H) and BrdU incorporation (Fig 3.9I) as well as cell proliferation (Supplementary Figure 3.VI). Together, these results confirm that β_2AR stimulation is capable of attenuating mitogen-induced H9C2, MEF and mESC proliferation.

Discussion

In this study, we have identified a novel mechanism regulating IR-induced ERK MAPK signaling with a preassembled IR/β_2AR complex (Fig 3.10). This is in contrast to the classic shc/grb2/SOS/ras/raf/MEK/ERK axis, which is activated at low concentrations of insulin to induce a nuclear ERK signal for gene expression. Here, we characterize an alternative, grb2/SOS-independent pathway[47] that is activated at high concentration of insulin. Activation of IR leads to IRS2-mediated GRK2 recruitment to the preassembled IR/β_2AR signaling complex, and promotes β_2AR phosphorylation on the cytoplasmic carboxyl terminus at serines 355 and 356. Upon GRK-mediated phosphorylation, β_2AR recruits β arrestin 2 for receptor internalization. The activated β_2AR/β arrestin 2 complex promotes full activation of ERK that is sequestered within the cytoplasm. This is the first evidence suggesting that an RTK can utilize a GPCR machinery to not only stimulate mitogenic signaling but also dictate the spatial profile of the signal.

Emerging evidence reveals that RTKs and GPCRs cross-communicate with each other, in particular, a growing list of GPCRs show capability in transactivation of RTKs[11, 34-39]. The full potential of this receptor cross talk on cellular physiology has not yet been realized. Similarly, a growing list of signal proteins which classically function in GPCR signaling pathways have been implicated in RTK signaling cascades, including G proteins[2, 3, 40, 48], GRKs[4, 5] and β arrestins[6-8, 41-43, 49]. For example, expression of a constitutively active Gαi2 (Q205L) promotes glucose transport and translocation of GLUT4 to the plasma membrane upon insulin stimulation[48]. IRs have also been shown to recruit GRKs[4, 5] and the scaffold/adaptor protein β arrestins[6-8] to regulate the GLUT4 dependent glucose uptake. Furthermore, down-regulation of β arrestin 1 by insulin treatment impairs MAP kinase signaling by both GPCRs and RTKs[49]. Meanwhile, numerous studies have shown that IR activation leads to βAR phosphorylation[9-11, 13, 14, 39]. The intimate relationship between IR and βAR prompts the search of an integrated machinery to coordinate activation of an array of different signaling pathways. One could speculate that IR and βAR function in a signalosome, like many receptor-receptor interactions[9-14, 32, 50]. In supporting this notion, we find that IR and β_2AR are preassembled into a complex on the plasma membrane in non-stimulated cells, consistent with the proximal membrane distribution of both the insulin receptor[51] and β_2AR[52] in lipid rafts.

Mechanistically, upon stimulation with insulin at high concentrations, IR leads to IRS2-dependent recruitment of GRK2, which is different from the previously reported G protein-dependent recruitment of GRK2[4], but is consistent with recently reported interaction between IRS1 and GRK2 in cardiac myocytes[5]. The recruited GRK2 promotes phosphorylation of the β_2AR at serines 355 and 356 in the IR-β_2AR complexes; and this GRK2 could also potentially phosphorylate IRS2 for attenuation of insulin signaling[5]. The phosphorylated β_2AR recruits β

arrestin 2 for receptor internalization. Interestingly, IR also associates with β arrestin 2 at basal conditions (Fig 3.3 and[45]). Upon stimulation, however, IR dissociates from both β_2AR and β arrestin 2, probably for further attenuation of insulin stimulated IR signaling. Thus, while the bound β_2AR/β arrestin 2 complexes undergo internalization, the dissociated IRs either remains at the cell surface or undergo internalization via different pathway[53]. In addition, several other residues on the carboxyl terminus of the β_2AR have also been characterized as substrates for insulin-induced PI3K/AKT activity[13], as well as IR tyrosine kinase and Src activities[14, 50], which could also contribute to receptor internalization. The activated β_2AR/β arrestin 2 complex is required for full increase of ERK activities under insulin stimulation, similar to the role of β_2AR/β arrestin 2 complex in promoting ERK activation under β_2AR stimulation[30, 31]. Furthermore, β arrestin 2 scaffold formation offers a unique mechanism of regulating ERK signaling by facilitating activation and cytoplasmic retention[42, 54], thus decreasing transcription in the nucleus[55].

Thus, stimulation of IR with insulin is capable of activating two alternate mitogenic signaling pathways for distinct subcellular distribution and cellular responses. The first pathway, activated by the classical Shc/Grb2/SOS/Ras/Raf/MEK axis[24], is stimulated at low insulin concentrations (≤10 nM). Activation of this pathway leads to nuclear translocation of ERK and promotes cellular proliferation via activation of transcription factors including Elk-1[56] and GATA-4[57]. The second pathway, activated by higher insulin concentrations, requires β_2AR expression for formation of the activated β_2AR/β arrestin 2 scaffold to promote full ERK activation. Activation of this second pathway prevents nuclear accumulation of ERK and attenuates cellular proliferation. Deletion of either β_2AR or β arrestin 2 significantly reduced the insulin-induced ERK activities, and allows nuclear ERK translocation and promotes cellular

proliferation. While nuclear targeting of ERK is necessary for transcription[55], cytoplasmic retention may allow targeting to non-nuclear ERK substrates involved in different cellular stress processes[58], receptor desensitization[59] and protein synthesis (Fig 3.8 and [55]).

Interestingly, we observe that nuclear translocation of ERK upon low dose insulin stimulation does not occur upon concomitant β_2AR stimulation. This is consistent with observations that the β_2AR/β arrestin 2 scaffold is capable of sequestering ERK activated by other GPCRs. This sequestration attenuates cellular proliferation[1], and β arrestin-mediated ERK retention within the cytoplasm prevents ERK-mediated transcription[55]. As a result, β_2AR stimulation prevents insulin-induced cell proliferation as well as protein synthesis. Considering the ability of numerous RTKs to recruit β arrestins[6-8, 41-43, 49], our data suggest a general mechanism of β arrestin 2-mediated βAR modulation of mitogenic signaling upon RTK activation with broad implications in physiological responses upon hormonal regulation *in vivo*. It also underscores the potent inhibitory effects of adrenergic signaling on tissue development and regeneration. Surprisingly, β_2AR deletion only attenuated insulin- and IGF-1-mediated ERK activation while stimulation with other RTK ligands including EGF, FGF, and PDGF was unaffected by β_2AR deletion. Thus, the cross talk with the β_2AR seems to be specific to class II RTKs, including IR and IGFR. Accordingly, concomitant βAR stimulation attenuates IGF-1 mediated self-renewal of mouse embryonic stem cells, indicating that βAR-mediated attenuation of class II RTK-induced cellular proliferation is universal mechanism.

In summary, we have characterized a novel mechanism in which IR is capable of transactivating the β_2AR via GRK phosphorylation leading to β arrestin 2-dependent activation and modulation of IR induced mitogenic signaling. RTKs utilization of a GPCR machinery to induce subcellular activation of mitogenic signaling presents a novel paradigm in RTK signal

transduction. Our data also provide the first evidence showing that β arrestin 2 acts as a coordinator to integrate subcellular mitogenic signals between two major cell surface receptors, RTKs and GPCRs, underscoring the critical role of signaling cross talk among GPCRs and RTKs *in vivo*.

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Figures & Figure Legends

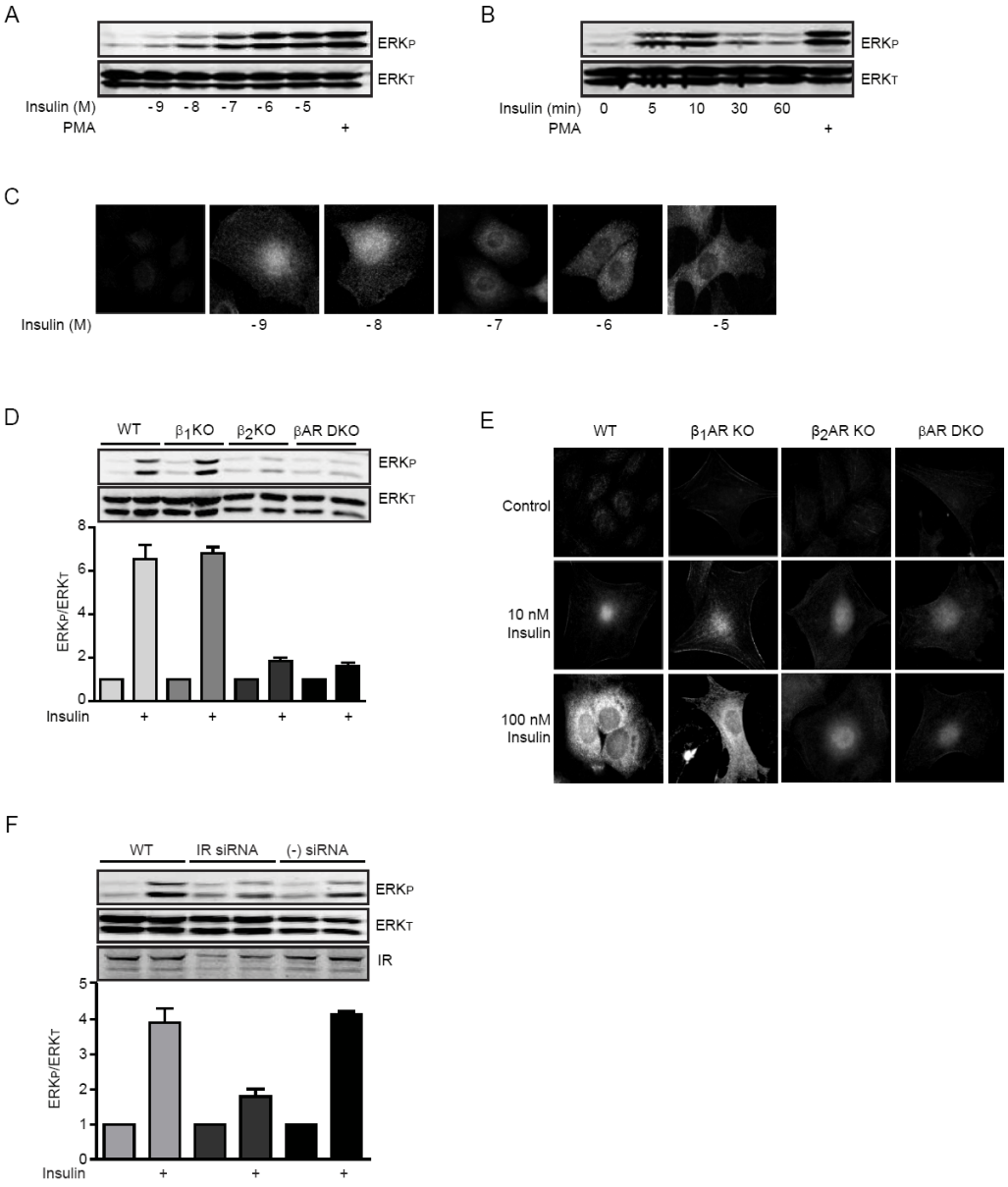


Figure 3.1. β_2AR is required for fine-tuning IR-induced subcellular ERK signaling A, WT MEF cells were treated with either insulin at the indicated concentration or PMA at 1 μM for 10 mins. Phospho-ERK (ERK_P) and total ERK (ERK_T) were then detected by Western blot. B, WT MEF cells were treated with 100 nM insulin for the indicated times or PMA (1 μM) for 10 mins. Cells were lysed for SDS PAGE. ERK_P and ERK_T were then detected by Western blot. C, WT MEF cells were treated with insulin at the indicated concentrations for 10 mins. Cells were then fixed for ERK_P staining. D, WT, β_1AR KO, β_2AR KO, β_1AR/β_2AR DKO MEF cells were stimulated with 100 nM insulin for 10 mins. Cells were lysed for SDS PAGE. ERK_P and ERK_T were then detected by Western blot. ERK_P was normalized against ERK_T. E, WT, β_1AR KO, β_2AR KO or βAR DKO cells were stimulated with either 10 nM or 100 nM insulin for 10 mins. and fixed for ERK_P staining. F, WT MEF cells were transfected with a siRNA oligo targeting the mouse insulin receptor. A scramble siRNA oligo was used as a negative control. Cells were stimulated with 100 nM insulin for 10 mins. Cells were then lysed and ERK_P, ERK_T and insulin receptor (IR) were then detected by Western blot. ERK_P was normalized against ERK_T.

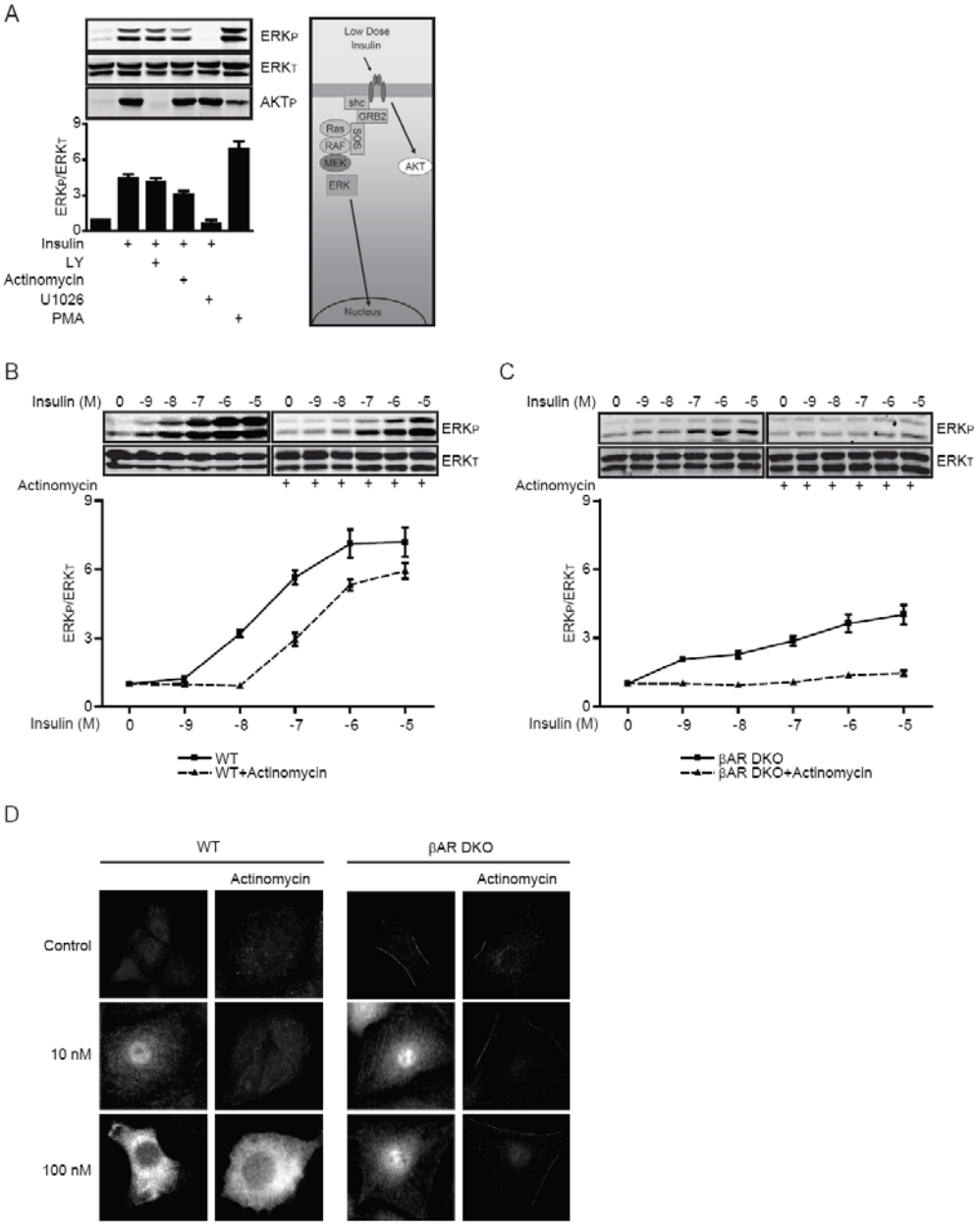


Figure 3.2. Cross talk with the β_2AR is a significant pathway contributing to IR-induced ERK signaling A, H9C2 cardiac myoblasts were pretreated with either the AKT inhibitor LY294002 (LY; 10 μM, 10 mins.), Shc/Grb2 inhibitor Actinomycin D (Actinomycin; 100 nM, 1h), MEK inhibitor U1026 (10 μM, 30 mins.) or the PKC stimulator PMA (1 μM, 10 mins.). Cells were then stimulated with 100 nM insulin for 10 mins. Phospho-ERK (ERK_P) and total ERK (ERK_T) and phospho-AKT (AKT_P) were then detected by Western blot. ERK_P was normalized against ERK_T. WT (B) or βAR DKO (C) MEF cells were pretreated, if indicated, with Actinomycin D (Actinomycin; 100 nM, 1h) and then stimulated with the indicated concentration of insulin for 10 mins. ERK_P and ERK_T were then detected by Western blot. ERK_P was normalized against ERK_T. D, WT or βAR DKO MEF cells were pretreated, if indicated, with Actinomycin D (Actinomycin; 100 nM, 1 hr.) and then stimulated with either 1 nM or 100 nM for 10 mins. Cells were then fixed for ERK_P staining.

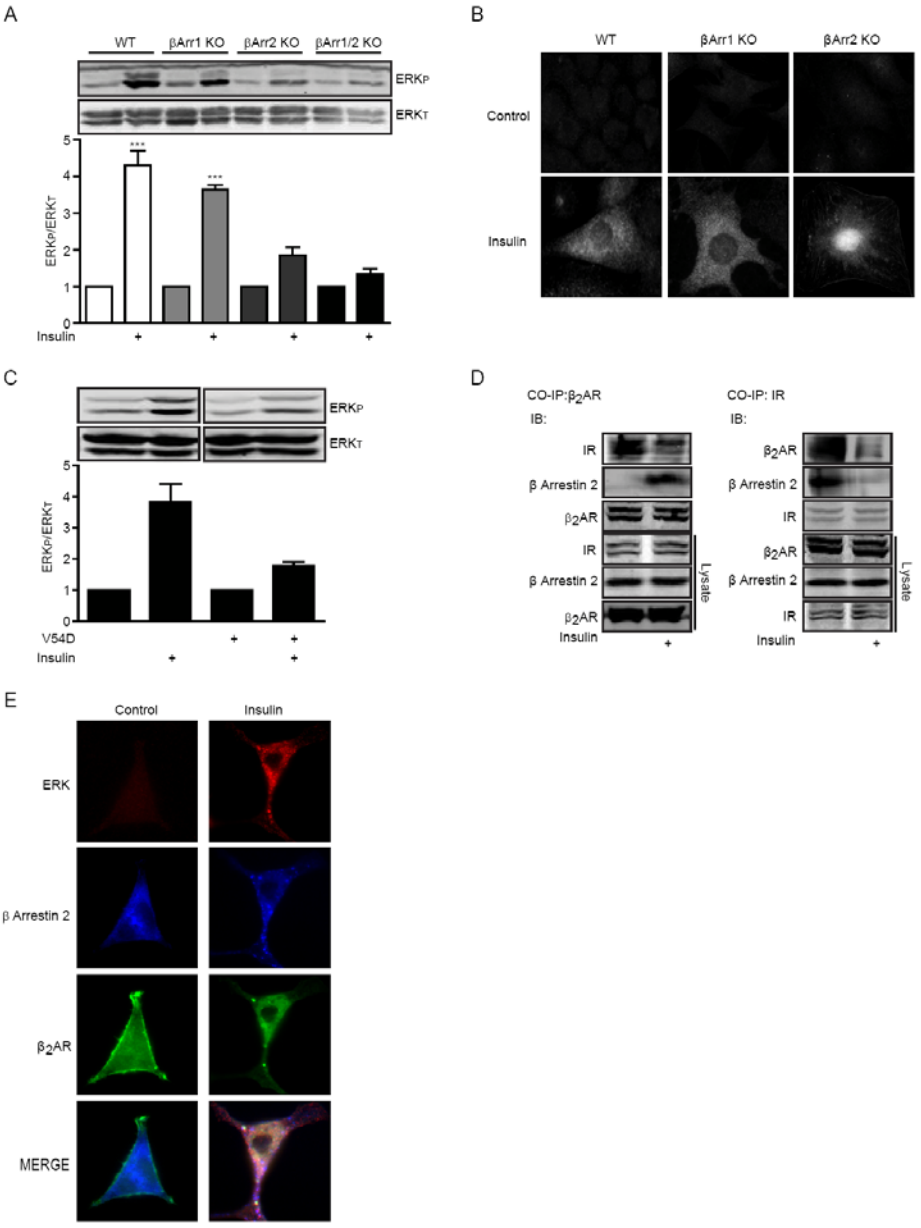


Figure 3.3. β_2AR-dependent cytoplasmic sequestration of insulin-induced mitogenic signaling occurs via β_2AR/β arrestin 2 scaffold formation A, WT, β arrestin 1 KO (βArr1 KO), β arrestin 2 KO (βArr2 KO) and β arrestin 1/β arrestin 2 DKO (βArr1/2 KO) MEF cells were stimulated with 100 nM insulin for 10 mins. Phospho-ERK (ERK_P) and total ERK (ERK_T) were detected by Western blot. ERK_P was normalized against ERK_T. B, WT, β arrestin 1 KO or β arrestin 2 KO MEF cells were stimulated with 100 nM insulin for 10 mins. and fixed for ERK_P staining. C, H9C2 cells were transfected for 48h with a mutant β arrestin 2 (V54D) that is incapable of internalizing the β_2AR. Cells were then stimulated with 100 nM insulin for 10 mins. ERK_P and ERK_T were then detected by Western blot. ERK_P was normalized against ERK_T. D, H9C2 cardiac myoblasts expressing FLAG-β_2AR together with GFP-β arrestin 2 were stimulated with 100 nM insulin for 10 mins. Cells were lysed for immunoprecipitation with either an anti-β_2AR (M2) or an anti-IRα antibody to examine the association between the β_2AR/IR/β arrestin 2 complex. E, H9C2 cardiac myoblasts expressing GFP-β arrestin 2 and FLAG-β_2AR were stimulated with 100 nM insulin for 10 mins. before being fixed for either ERK_P, β_2AR or β arrestin 2 staining.

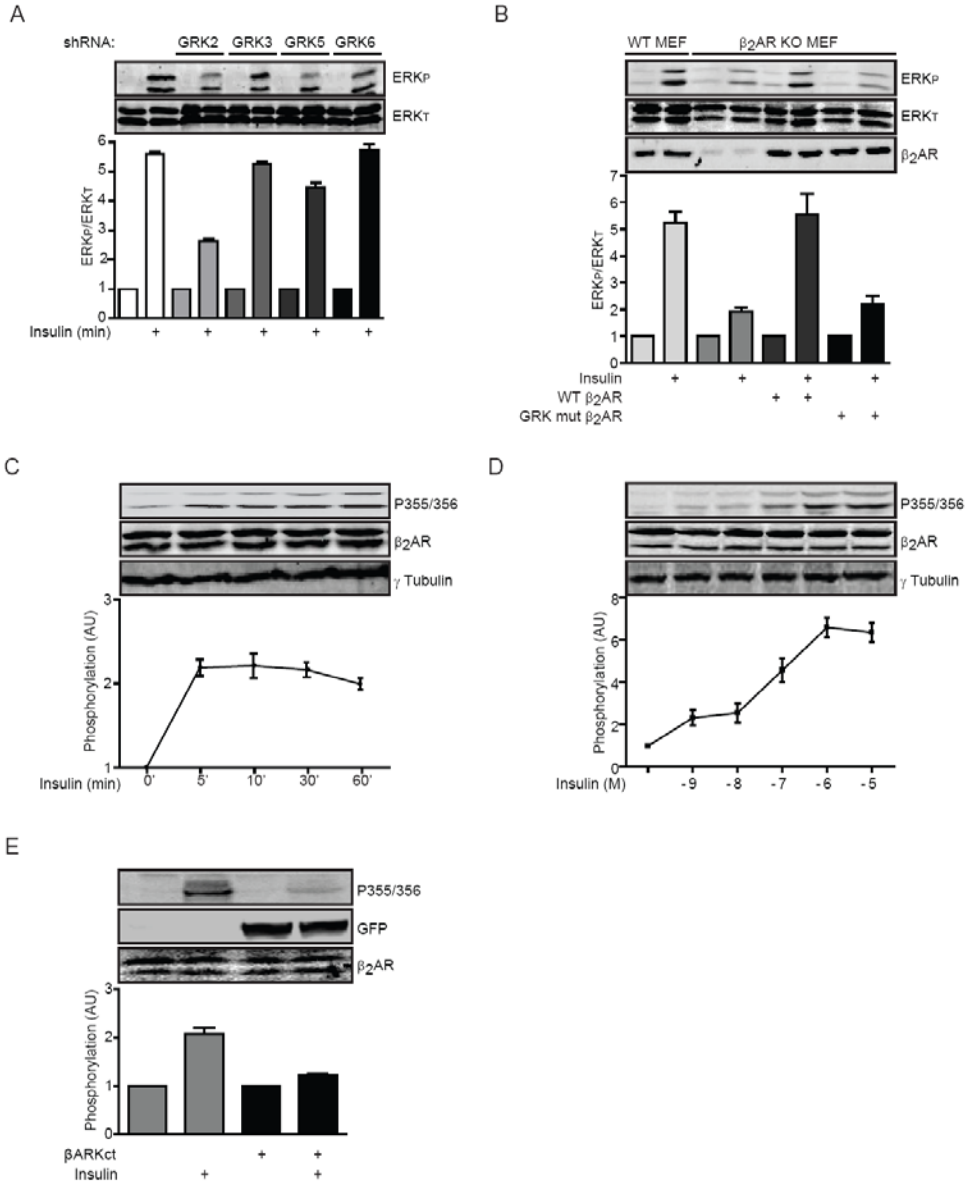


Figure 3.4. GRK2-mediated β_2AR phosphorylation is required for insulin-induced ERK activity A, WT MEF cells were infected with lentiviruses expressing GRK-specific shRNA constructs for 72h with puromycin selection. Cells were then stimulated with 100 nM insulin for 10 mins. Phospho-ERK (ERK_P) and total ERK (ERK_T) were then detected by Western blot. ERK_P was normalized against ERK_T. B, β_2AR KO MEF cells were transfected with WT β_2AR or a β_2AR with mutated GRK phosphorylation sites (GRK mut β_2AR). WT and β_2AR KO MEF cells were then stimulated with 100 nM insulin for 10 mins. ERK_P, ERK_T and total β_2AR were then detected by Western blot. ERK_P was normalized against ERK_T. H9C2 cardiac myoblast cells were infected with adenovirus expressing mouse β_2AR and then stimulated with 100 nM insulin for the indicated times (C) or for 10 mins. at the indicated dose (D). GRK-mediated phosphorylation of the β_2AR at serines 355/356 was detected by Western blot. 355/356 phosphorylation was normalized against total β_2AR and represented as arbitrary units (AU). E, H9C2 cells were infected with adenoviruses expressing either mouse β2AR and/or βARKct for 48h. Cells were then stimulated with 100 nM insulin for 10 mins. GRK-mediated phosphorylation of the β_2AR at serines 355/356 in addition to total β_2AR and GFP were detected by Western blot. Serines 355/356 phosphorylation was normalized against total β_2AR and represented as arbitrary units (AU).

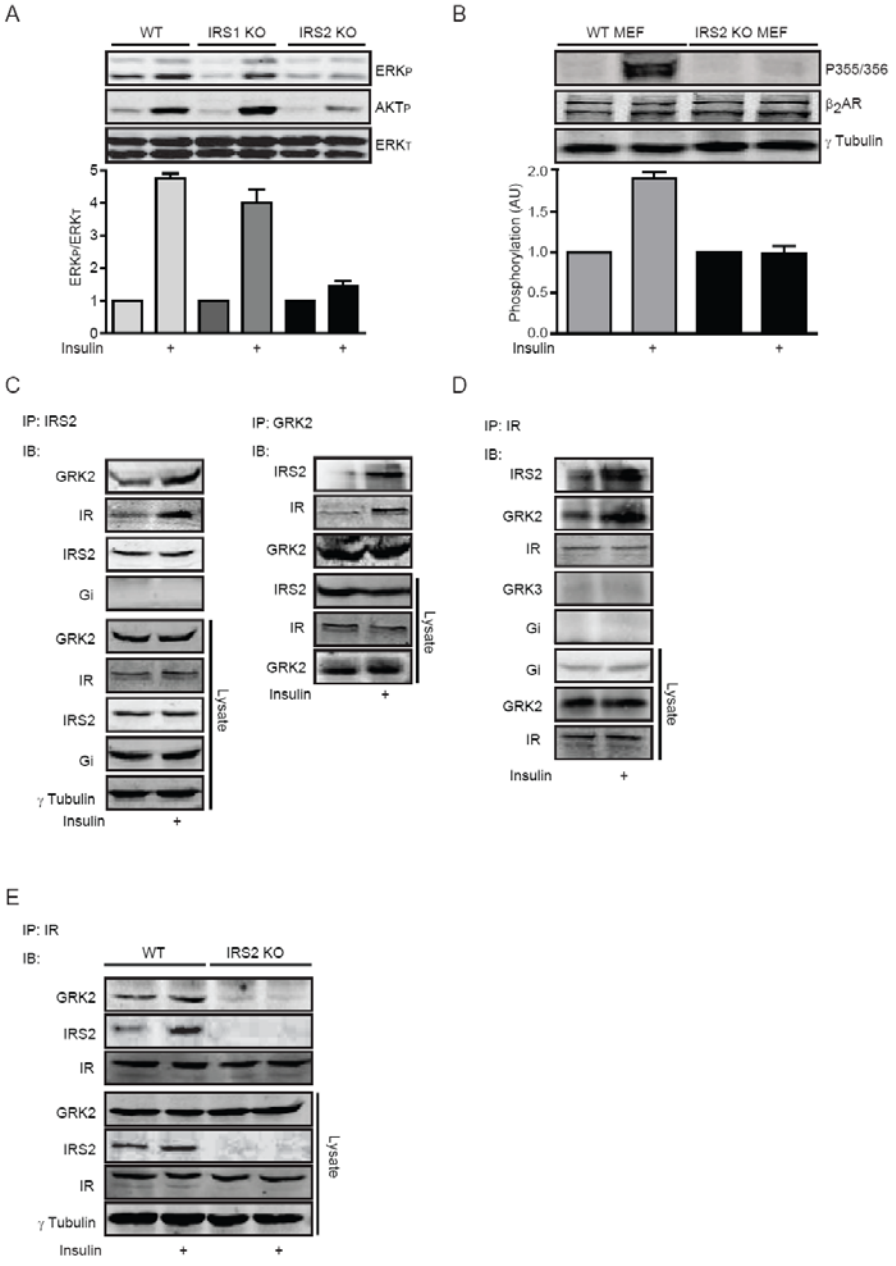


Figure 3.5. IRS2 recruits GRK2 to the insulin receptor allowing β_2AR phosphorylation A, WT, insulin receptor substrate 1 knock out (IRS1 KO) or insulin receptor substrate 2 knock out (IRS2 KO) MEF cells were stimulated with 100 nM insulin for 10 mins. Phospho-ERK (ERK_P), total ERK (ERK_T) and phospho-AKT (AKT_P) were then detected by Western blot. ERK_P was normalized against ERK_T. B, WT or IRS2 KO MEF cells were transfected with mouse β_2AR and stimulated with 100 nM insulin for 10 mins. GRK-mediated phosphorylation of the β_2AR at serines 355/356 in addition to total β_2AR was detected by Western blot. Serine 355/356 phosphorylation was normalized against total β_2AR and represented as arbitrary units (AU). C, WT MEF cells were lysed for immunoprecipitation with either an anti-IRS2 antibody or an anti-GRK2 antibody upon insulin stimulation (100 nM, 10 mins.). D, WT MEF cells were lysed for immunoprecipitation with an anti-insulin receptor α (IR) antibody upon 100 nM insulin stimulation for 10 mins. E, WT or IRS2 KO MEF cells were lysed for immunoprecipitation with an anti-IR antibody upon 100 nM insulin stimulation for 10 mins.

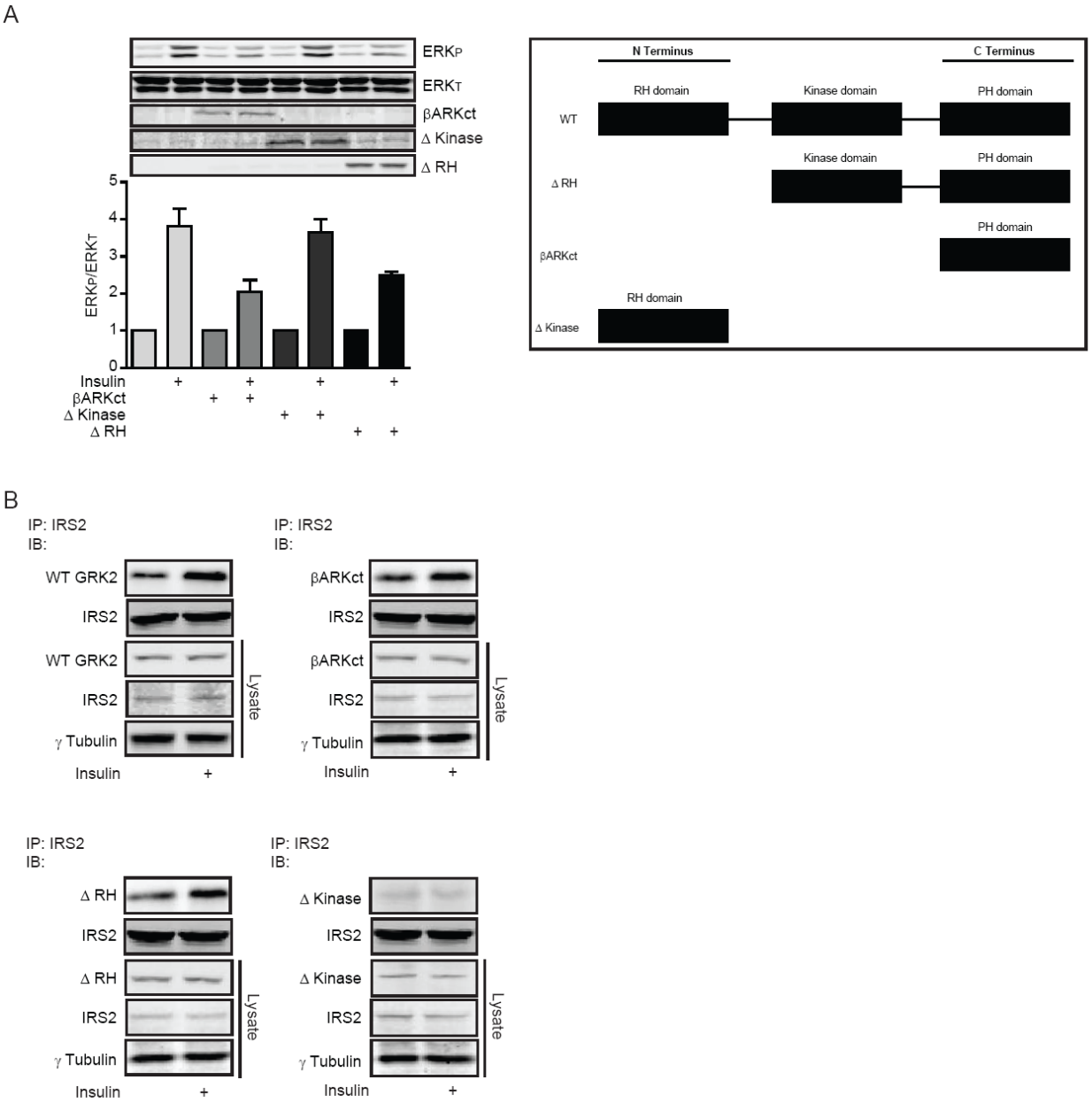


Figure 3.6. The C-terminal Pleckstrin Homology domain is essential in recruiting GRK2 to IRS2 A, WT MEF cells were transfected with plasmids expressing a polypeptide inhibitor of GRK2 (βARKct) lacking both the Kinase and N terminal RH domains, mutant GRK2 with a kinase domain mutation (Δ kinase), or a mutant GRK2 with an N terminal RGS homology domain deletion (Δ RH) for 48 hrs. Mutants are represented in the adjacent model. Cells were then stimulated with 100 nM insulin for 10 mins. in the presence or absence of these mutant GRK2 constructs. ERK_P, ERK_T and HA (Δ RH and Δ Kinase) or GFP (βARKct) were then detected by Western blot. ERK_P was normalized against ERK_T. B, WT MEF cells were transfected with either WT GRK2, a GFP-tagged βARKct construct, a HA-tagged ΔRH construct, or the Δ Kinase mutant. Cells were stimulated with 100 nM insulin for 10 mins. Cells were then lysed for immunoprecipitation with an anti-IRS2 antibody. Proteins were resolved via SDS-PAGE and blotted as indicated.

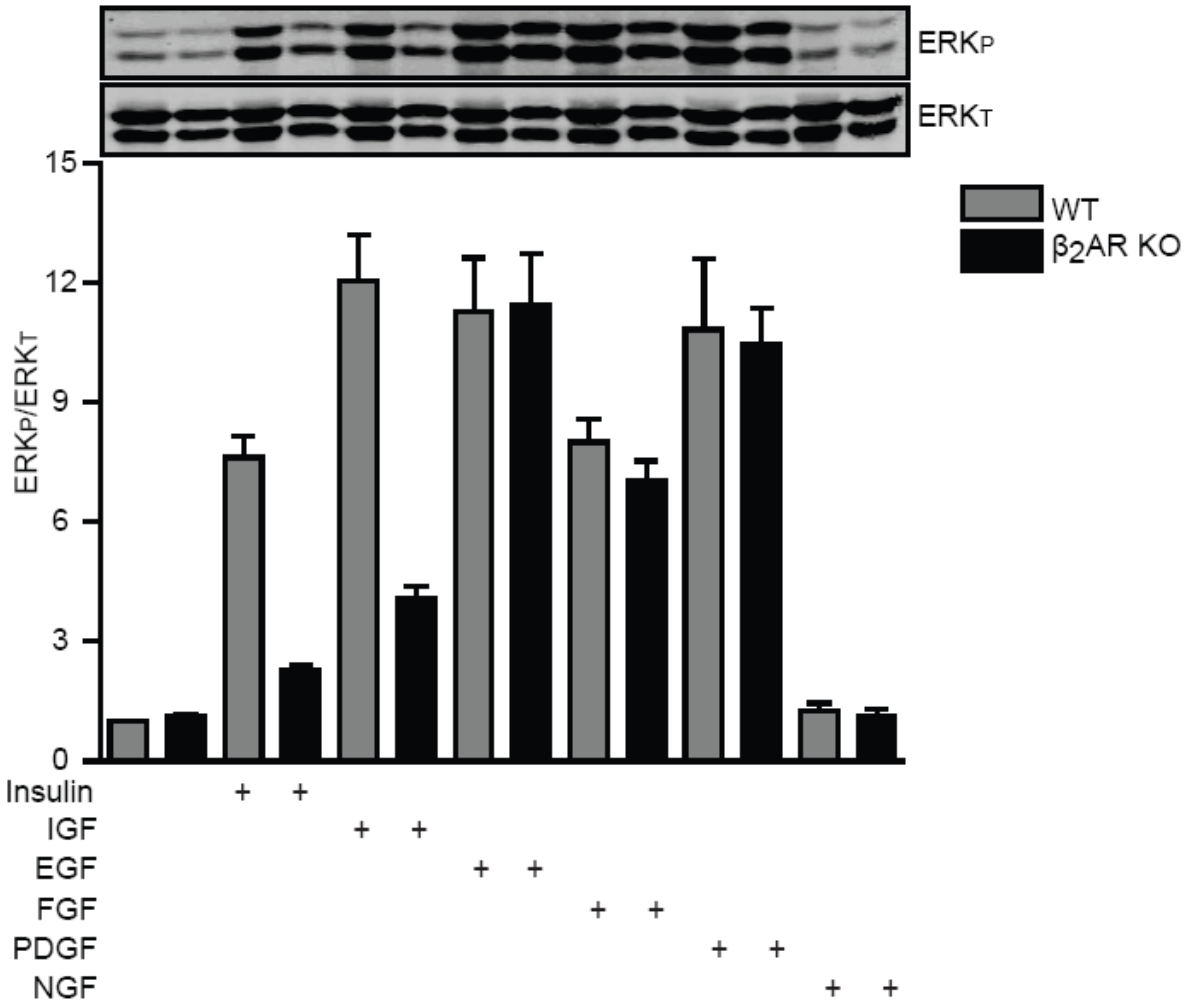


Figure 3.7. ERK activation upon cross talk with the β_2AR is specific to type 2 RTKs WT or β_2AR KO MEF cells were stimulated with insulin (100 nM, 10 mins.), insulin-like growth factor (IGF; 100 nM, 10 mins.), epidermal growth factor (EGF; 100 nM, 10 mins.), fibroblast growth factor (FGF; 100 nM, 10 mins.), platelet-derived growth factor (PDGF; 100 nM, 10 mins.) or nerve growth factor (NGF; 100 nM, 10 mins.). Cells were then lysed and harvested for Western blot. Phospho-ERK (ERK_P) and total ERK (ERK_T) were then detected by Western blot. ERK_P was normalized against ERK_T.

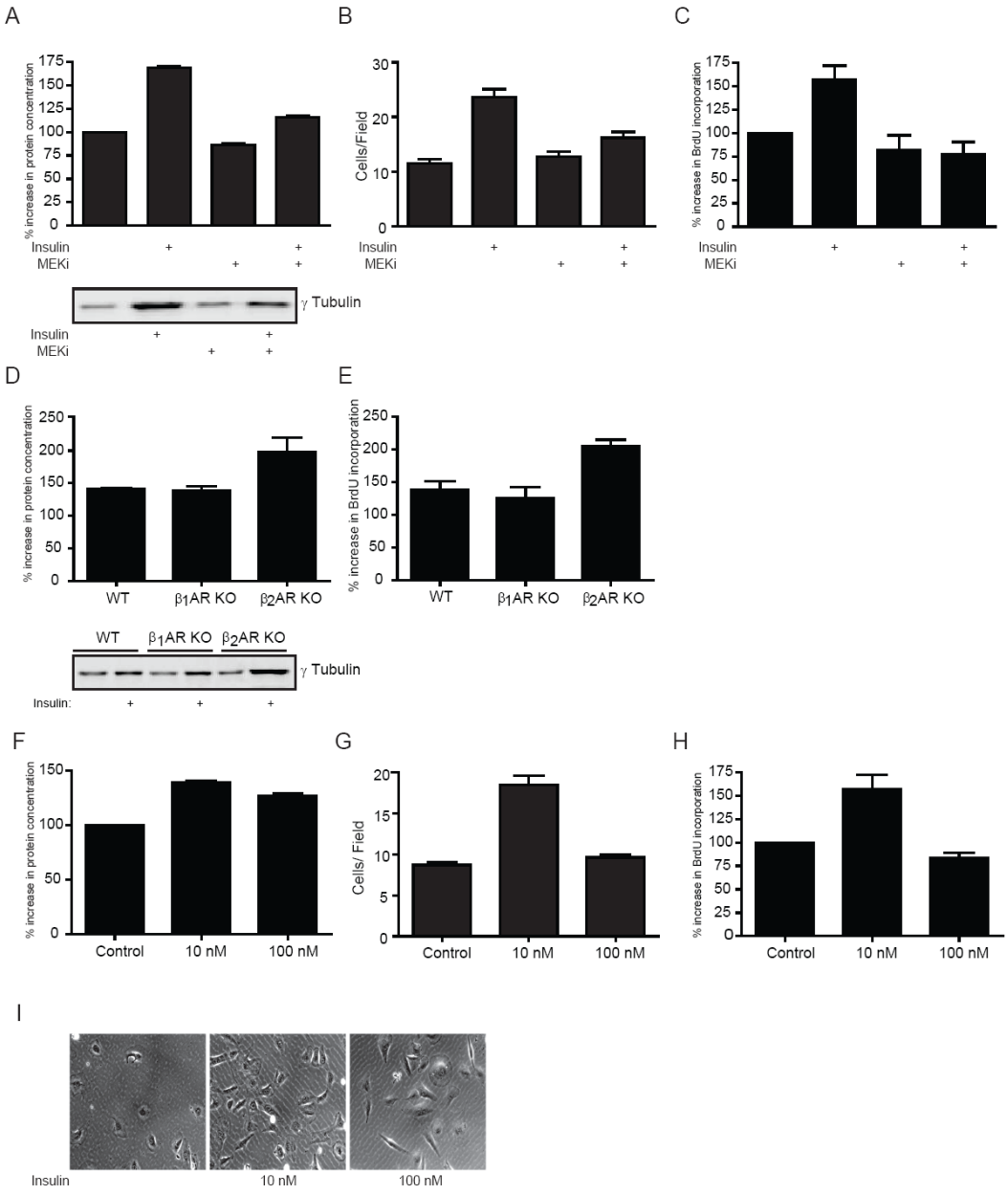


Figure 3.8. ERK dependent cell proliferation is insulin dose-dependent and is attenuated by the β_2AR H9C2 cells were pretreated with 1 μM of the MEK inhibitor U1026 (MEKi) for 30 mins. Cells were then stimulated with 10 nM insulin for 48 hrs. A, Cells were processed for quantitation of total protein concentration via BCA assay. In addition, equal volumes of lysates were resolved via SDS-PAGE and blotted for γ-tubulin. Alternatively, cells were either counted in a blinded manner (B), or BrdU incorporation was measured, and the data were normalized against the control (C). WT, β_1AR KO or β_2AR KO MEF cells were stimulated with 100 nM insulin for 48h cells were then lysed and harvested. Total protein concentration was measured via BCA assay (D). Equal volumes of lysates were resolved via SDS-PAGE and blotted for γ tubulin. Alternatively, BrdU incorporation was measured, and the data were normalized against the control (E). H9C2 cardiac myoblasts were treated for 48h with either 10 nM or 100 nM insulin and total protein concentration was quantitated via BCA assay (F). Alternatively, cells were either counted in a blinded manner (G), or BrdU incorporation was measured, and the data were normalized against the control (H). In addition, cells were imaged 48 hrs. after 10 nM or 100 nM insulin treatment (I).

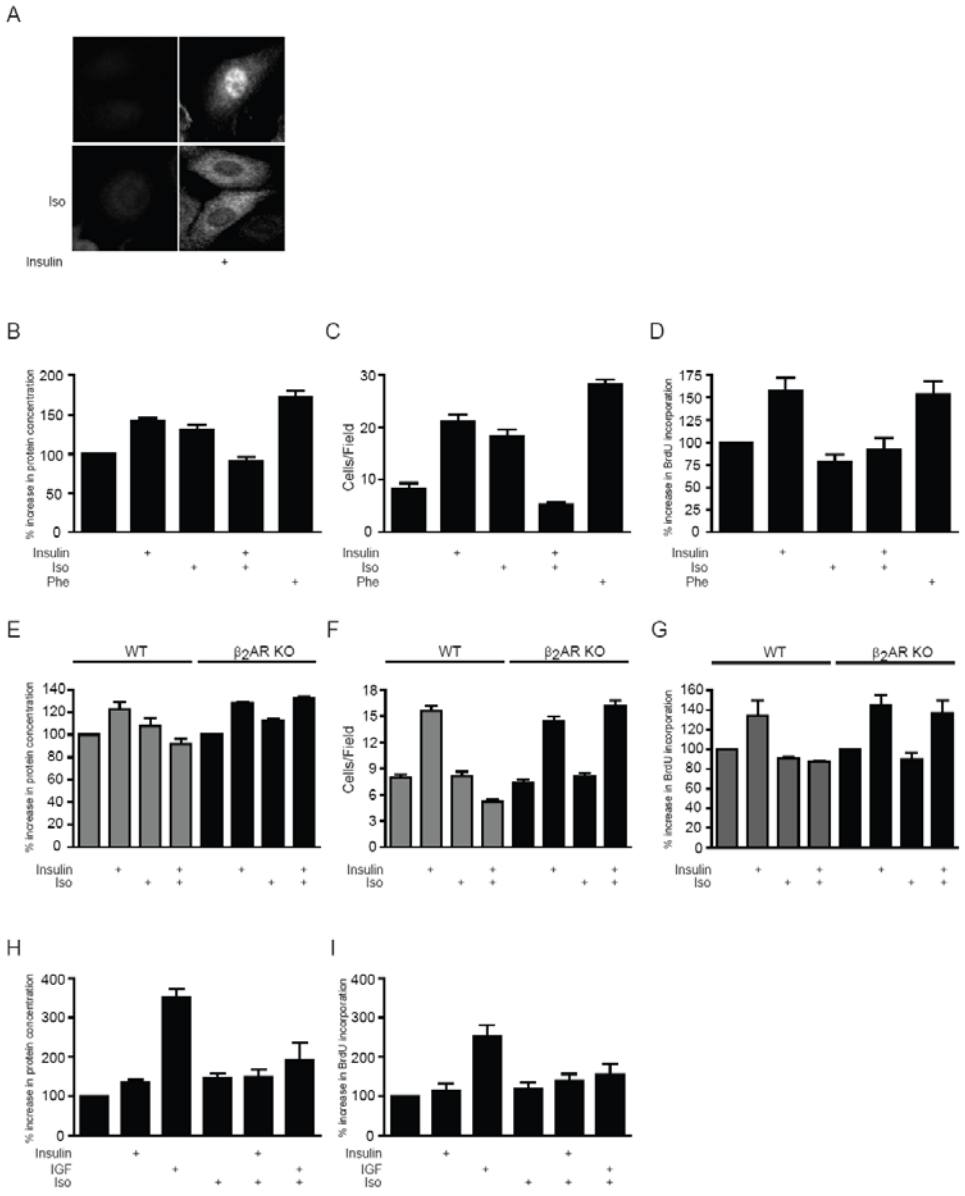


Figure 3.9. β_2AR activation redistributes RTK-induced ERK to the cytoplasm and prevents cell proliferation A, H9C2 cardiac myoblasts were pretreated, if indicated, with 10 μM isoproterenol for 10 mins. and stimulated with 10 nM insulin for 10 mins. Cells were fixed for phospho-ERK (ERK_P) staining. H9C2 cardiac myoblasts were preteated, if indicated, with isoproterenol (Iso, 10 μM, 10 mins.) and then stimulated for 48h with 10 nM insulin or 10 μM of the α1AR agonist phenylephrine (Phe). Total protein concentration was measured via BCA assay (B), cells were then counted in a blinded manner (C), and BrdU incorporation was measured, and the data were normalized against the control (D). WT or β_2AR KO MEF cells were pretreated, if indicated, for 10 mins. with isoproterenol (Iso) and then stimulated for 48h with 10 nM insulin. Cells were then processed for quantitation of total protein concentration via BCA assay (E). Alternatively, cells were counted in a blinded manner (F), or BrdU incorporation was measured, and the data were normalized against the control (G). WT mouse embryonic stem cells were pretreated for 30 mins. with 10 μM isoproterenol, if indicated, and then stimulated for 24h with insulin (100 nM) or insulin-like growth factor (IGF; 100 nM). Total protein concentration was quantitated via BCA assay (H). Alternatively, BrdU incorporation was measured, and the data were normalized against the control (I).

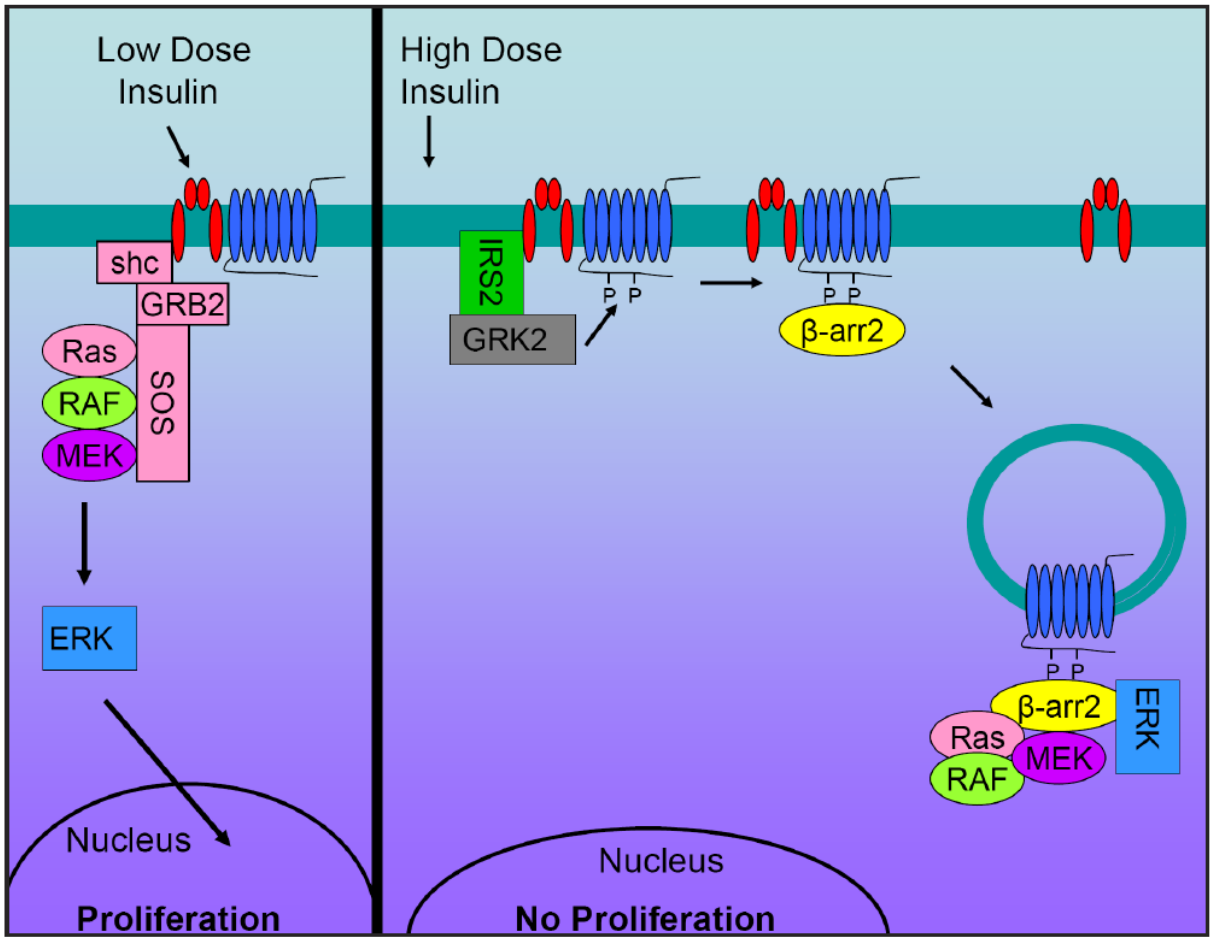
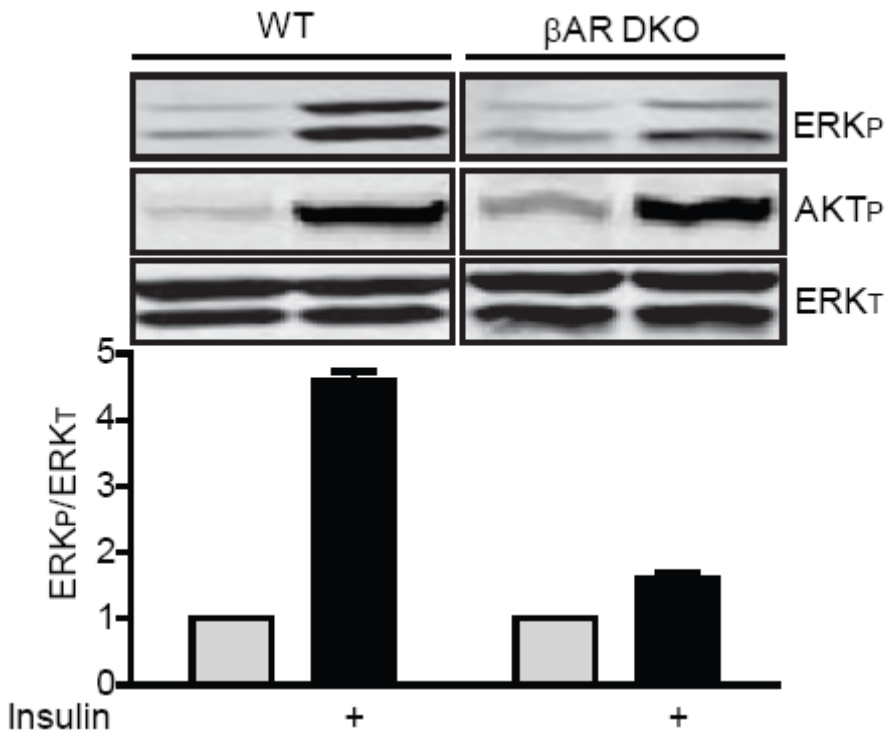
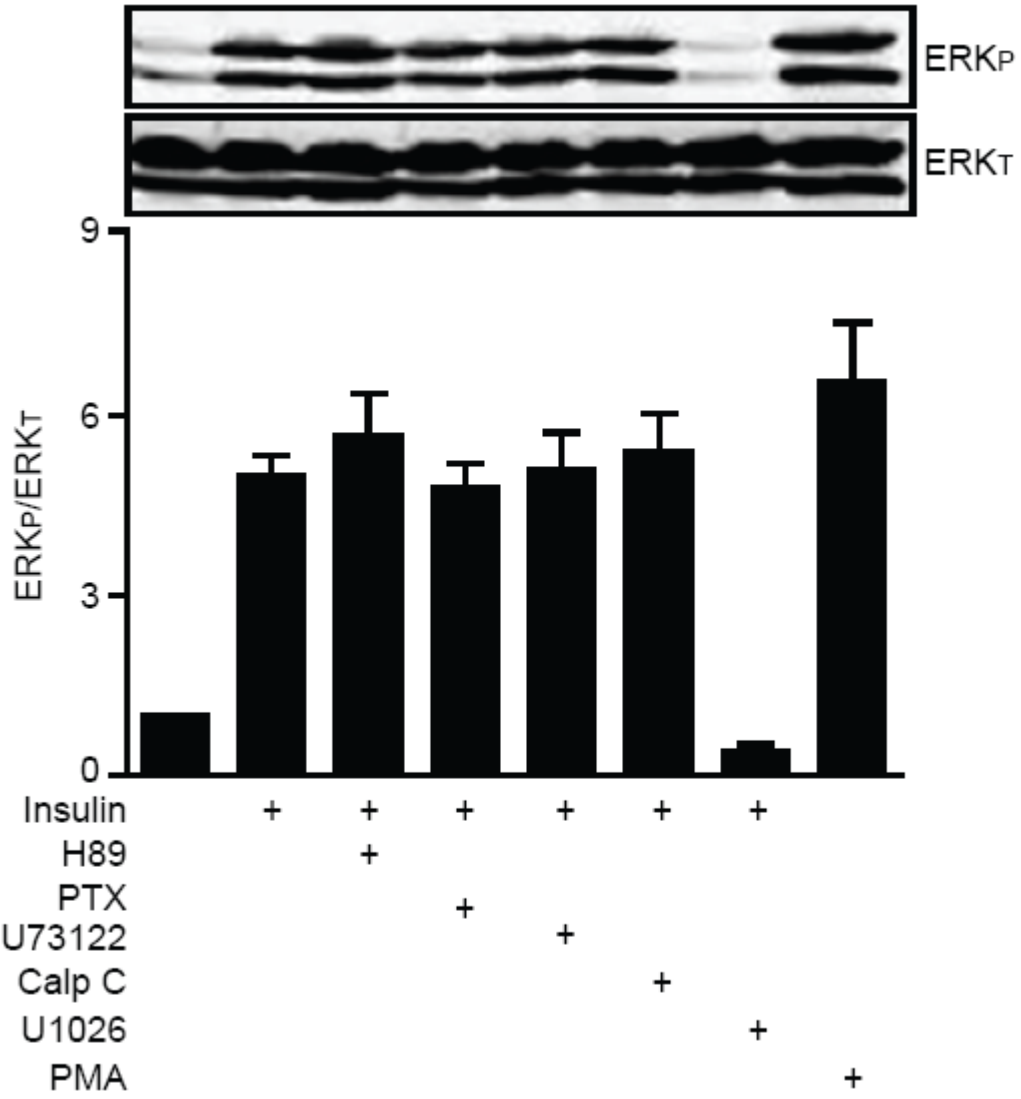


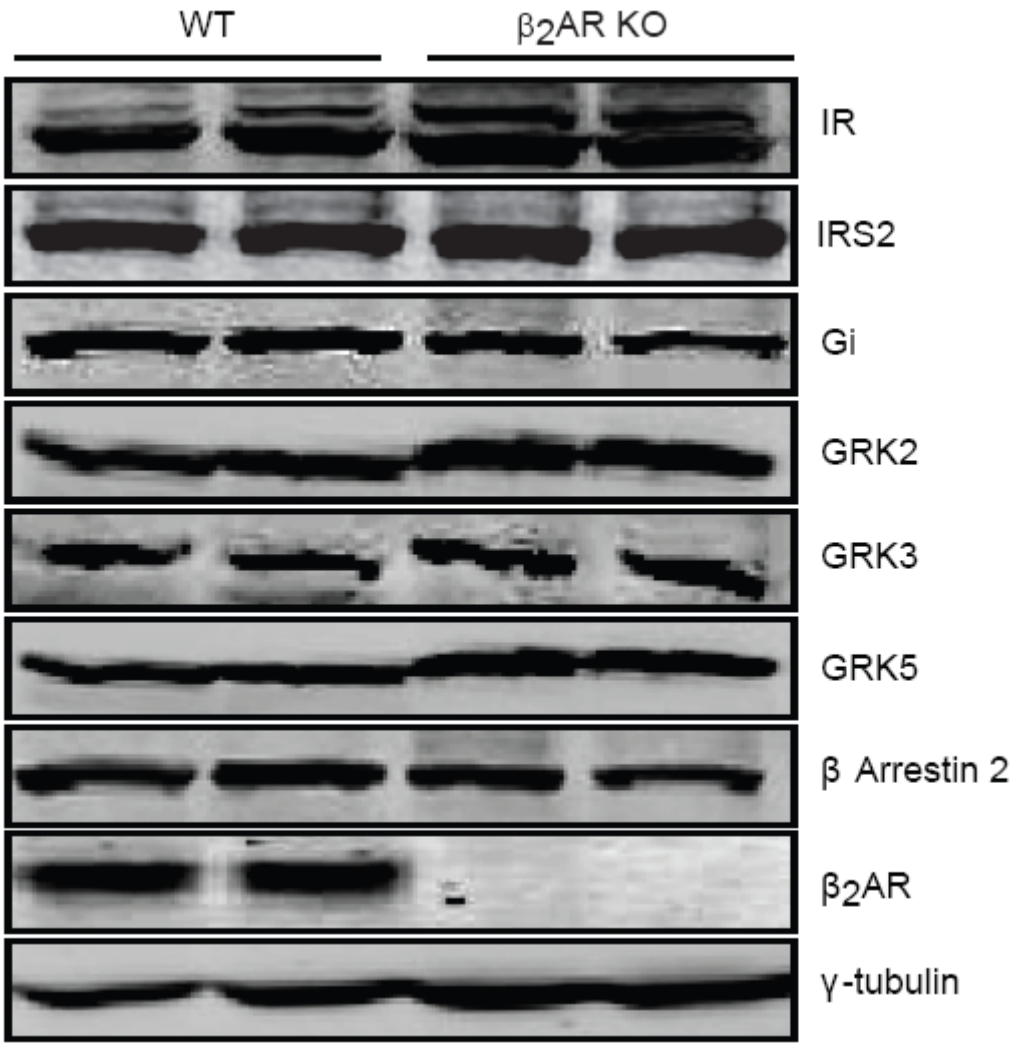
Figure 3.10. Model of the mechanism on β_2AR/β arrestin 2 complex mediated sequestration of insulin induced ERK activation At low concentrations of insulin (10 nM), ERK is activated via a classical IR/Shc/Grb2/SOS axis and translocates to the nucleus to promote cell proliferation. Alternatively, activation of the insulin receptor (IR) with high concentrations (100 nM and above) of insulin induces IRS2-mediated GRK2 recruitment to the IR, which allows phosphorylation of the β_2AR at serines 355/356. GRK2-mediated β_2AR phosphorylation leads to β arrestin 2 recruitment and internalization of the β_2AR. Formation of this β_2AR/β arrestin 2 signaling complex leads to ERK activation and is capable of sequestering phospho-ERK originating from the IR within the cytoplasm.



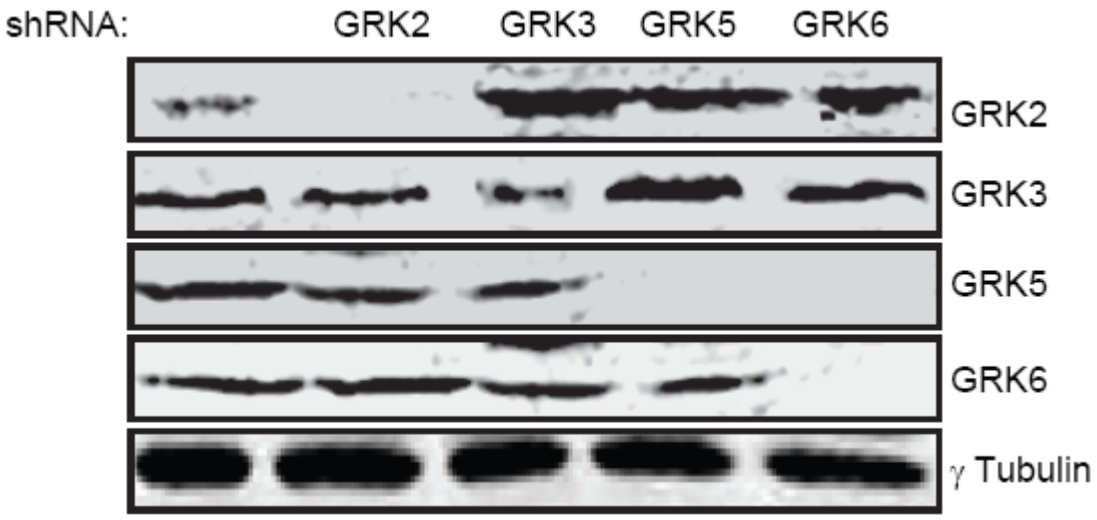
Supplementary Figure 3.I. βAR knock out alters ERK, but not AKT, signaling WT or β_1AR/β_2AR DKO (βAR DKO) MEF cells were stimulated with 100 nM insulin for 10 mins. phospho-ERK (ERK_P), total ERK (ERK_T) and phospho-AKT (AKT_P) were detected via Western blot.



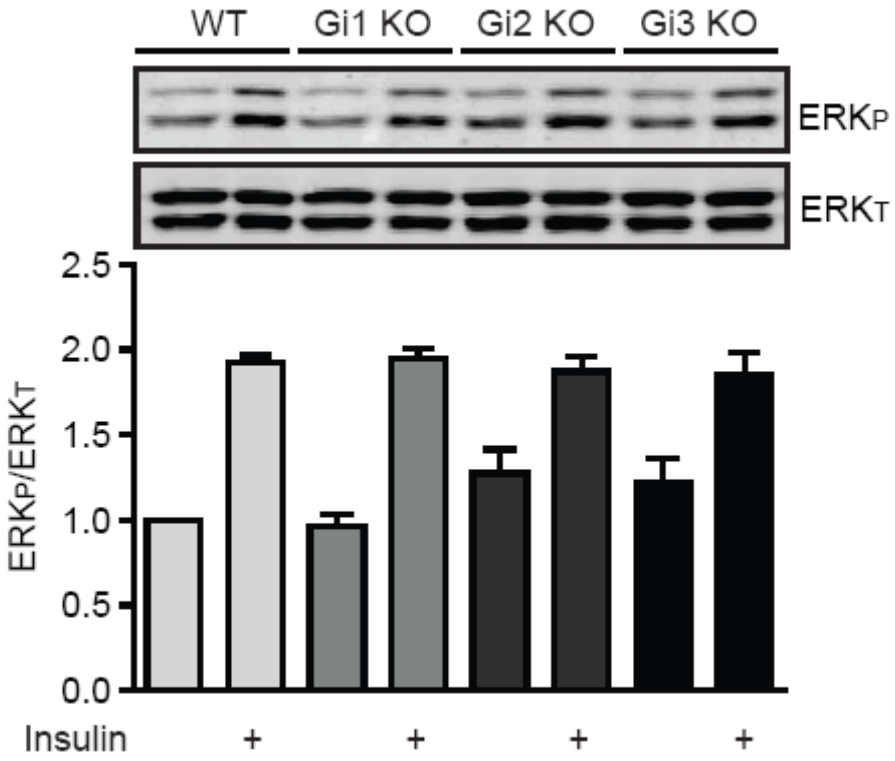
Supplementary Figure 3.II. IR cross talk occurs independently of classical β_2AR signaling pathways H9C2 cardiac myoblast cells were pretreated with either the PKA inhibitor H89 (10 μM, 30 mins.), the Gi inhibitor pertussis toxin (PTX; 200 nM, O/N), the PLC inhibitor U73122 (1 μM, 30 mins.), the PKC inhibitor calphostin C (Calp C; 10 μM, 30 mins.), the MEK inhibitor U1026 (10 μM, 30 mins.), or the PKC stimulator PMA (1 μM, 10 mins.). Cells were stimulated with 100 nM insulin for 10 mins. Cells were then lysed and phospho-ERK (ERK_P) and Total ERK (ERK_T) were then detected by Western blot. ERK_P was normalized against ERK_T.



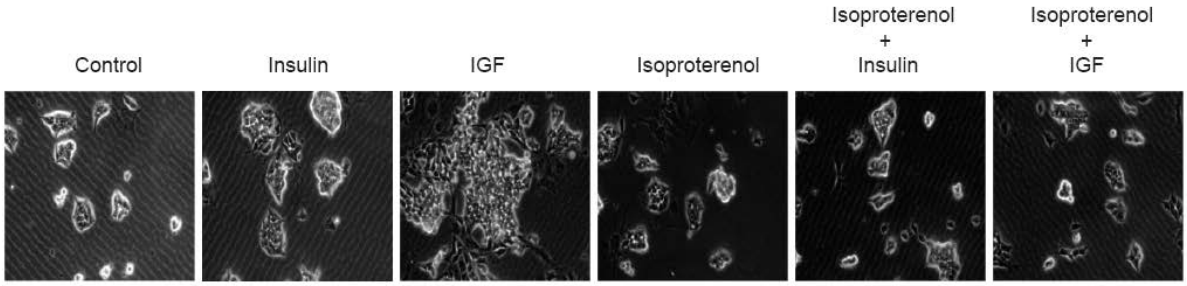
Supplementary Figure 3.III. Protein expression not altered in β_2AR MEF cells WT and β_2AR MEF cells were lysed and harvested for Western blot for the indicated proteins.



Supplementary Figure 3.IV. Successful knockdown of GRK isoforms in H9C2 cells H9C2 cells were infected with lentiviruses expressing the indicated GRK isoforms for 72h under puromycin selection. Cells were then lysed and harvested for Western blotting for the indicated GRK isoforms.



Supplementary Figure 3.V. Gi proteins are not required for cross talk between IR and β_2AR WT, Gi1 KO, Gi2 KO and Gi3 KO MEF cells were stimulated with 100 nM insulin for 10 mins. Cells were then lysed and phospho-ERK (ERK_P) and total ERK (ERK_T) were then detected by Western blot were then detected by Western blot. ERK_P was normalized against ERK_T.



Supplementary Figure 3.VI. β_2AR stimulation attenuates IGF-mediated mESC proliferation WT mouse embryonic stem cells were pretreated for 30 mins. with 10 μM isoproterenol, if indicated, and then stimulated for 24h with insulin (100 nM) or insulin-like growth factor (IGF; 100 nM). Images were taken after 24h.

Chapter 4:

Conclusions and future directions

Understanding how cells integrate information from a variety of chemically diverse signals into complex, orchestrated responses such as cell proliferation, differentiation and apoptosis is an overarching goal of cell biology. Adrenergic signaling within the heart represents essential signaling cascades regulating both cardiac performance as well as pathophysiological remodeling. Due to the complexities of hormonal signaling *in vivo,* it is essential to understand how concomitant activation of one signaling pathway can alter the physiologic outcome of another receptor's signaling. Here, I began characterize the cellular mechanism by which GPCR cross-talk regulates mitogen-activated protein kinase (MAPK) activation and defined how this regulation affects cellular proliferation. I then determined the mechanism by which type II RTK activation at high concentrations of mitogen recruits non-traditional GPCR signaling components to fine-tune activation of MAPK signaling. Lastly, I illustrated how this regulation affects cellular proliferation. These studies not only underscore the critical role of signaling cross-talk in the complex regulation of receptor signaling via sub cellular localization of signaling components, but also provide novel mechanisms into the regulation of mitogenic signaling elicited by different receptors. It is critical to understand the role of adrenergic receptor cross-talk in cardiac remodeling. With this understanding, more efficient pharmacologic agents may be developed to prevent the progression of this major health problem.

Although the β adrenergic receptors (ARs) are central to cardiac remodling within the heart, their primary role within the myocardium is regulation of heart rate and contractility. Moreover, the signaling pathways implicated in cardiac remodeling also are essential in controlling cadiac output. In addition, preliminary data from our lab suggests that cross-talk with

the insulin receptors is capable of blunting βAR-induced increases in cardiac contractility. The importance of adrenergic receptor cross talk in modulating cardiac performance remains an interesting, and physiologically relevant, topic for future research.